**University of Alberta**

# Insulin Receptor Translocation To The Hepatocyte Nucleus. Regulation Of Gene Expression

by

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of the requirements for the degree of Doctor of Philosophy

In

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled INSULIN RECEPTOR TRANSLOCATION TO THE HEPATOCYTE NUCLEUS. REGULATION OF GENE EXPRESSION submitted by NANA GLETSU in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in NUTRITION AND METABOLISM.

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# ABSTRACT

The objective of this research was to determine if ligand-induced insulin receptor internalization and translocation to the hepatocyte cell nucleus is a mechanism by which insulin regulates nuclear processes including gene expression. Eight week old male mice were fasted for 12 hours and then given an oral dose of glucose in order to stimulate hepatocytes with insulin *in vivo*. Serum concentrations of glucose and insulin were increased within 15 min of the glucose meal ($p < 0.05$). Insulin binding to isolated nuclei was increased by 205 ± 7 % compared to binding at basal conditions within 15 min following oral glucose. Peak serum insulin concentrations and insulin binding to isolated nuclei were correlated ($r^2 = 0.44$, $P < 0.01$), suggesting that increases in insulin levels induced insulin receptor translocation to the nucleus.

Ligand-induced translocation and internalization of insulin receptor to the hepatocyte nucleus was compared in lean versus obese mice following an oral glucose meal. Obese animals exhibited increased insulin receptor levels in the nucleus when measured by immunoassay compared to lean animals following an oral glucose meal ($p < 0.05$). This finding suggests that associated hyperinsulinemia causes increased insulin receptor levels in obese mice hepatocyte nuclei.

The effect of ligand-induced internalization and insulin receptor translocation to the nucleus on nuclear processes such as nuclear protein phosphorylation and insulin responsive gene expression was determined. Mice were fasted for 24 h and then given an

oral glucose meal. Insulin receptor levels and insulin receptor β-subunit autophosphorylation in the hepatocyte nucleus were increased by 81 % and by 150 % respectively, at 15 min following oral glucose ($p < 0.05$). The increase of activated insulin receptor in the cell nucleus was coincident with a decrease in the tyrosine phosphorylation state of a DNA-binding protein (31 % decrease compared to basal, $p < 0.05$), and an increase in mRNA levels of malic enzyme and glyceraldehyde 3-phosphate dehydrogenase (35 % increase and 16 % increase compared to basal, respectively, $p < 0.05$). These observations occurred within the time course of insulin receptor translocation to the nucleus, which suggests that changes in phosphorylation state of nuclear proteins and changes in insulin responsive gene expression may be regulated by insulin receptor in the cell nucleus.

Activated insulin receptor was incubated with nuclei isolated from 24 h-fasted animals and the effect on nuclear protein phosphorylation and changes in transcription factor binding to an insulin responsive element (IRE), within the malic enzyme gene, was determined. Insulin receptor attenuated the increase in phosphorylation of two DNA-binding proteins (78 and 73 % decrease compared to phosphorylation without receptor added, $p < 0.05$). Insulin receptor also increased the complex between transcription factors and the IRS within the malic enzyme promoter compared to that found in the basal state ($p < 0.05$).

These findings suggest a novel mechanism of insulin signal transduction by which insulin receptor may directly generate insulin signaling in the cell nucleus.

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# LIST OF ABBREVIATIONS

A = adenosine

ANOVA = analysis of variance

AntiIR = anti-insulin receptor antibody

AntiPY = anti-phosphotyrosine antibody

AP-1 = activator protein-1

ATP = adenosine 5'-triphosphate

BB = Biobreeding

C = cytidine

cAMP = cyclic adenosine 5'-monophosphate

CAT = chloroamphenicol-acetyl transferase

DNA = deoxyribonucleic acid

DTT = dithiothreitol

E = glutamate

EDTA = ethylenediaminetetraacetic acid

FAS = fatty acid synthase

G = guanosine

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

GLUT = glucose transporter

GRB-2 = growth factor receptor bound-2

GSK-3 = glycogen synthase kinase-3

GTP = guanosine 5'-triphosphate

HEPES = N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

IGF = insulin-like growth factor

IRE = insulin responsive element

IRS-1 = insulin receptor substrate-1

Kda = kilo dalton

M, Met = methionine

MAP-K = mitogen activated protein kinase

mRNA = messenger ribonucleic acid

N = asparagine

NAPDH = nicotinamide adenine dinucleotide phosphate

NIDDM = non-insulin dependant diabetes mellitus

P = proline

P/S = ratio of polyunsaturated fatty acid to saturated fatty acid

PBS = phosphate buffered saline

PC = phosphatidylcholine

PE = phosphatidylethanolamine

PEPCK = phosphoenolpyruvate carboxykinase

PI = phosphatidylinositol

P-I-3'K = phosphatidylinositol-3'kinase

PMSF = phenylmethylsulfonyl flouride

PUFA = polyunsaturated fatty acid

LCPUFA = long chain polyunsaturated fatty acid

SGLT-1 = sodium dependent D-glucose cotransporter

SDS-PAGE = sodium dodecyl sulfate polyacrylamide electrophoresis

SH2 = Src-homology 2

T = thymidine

# CHAPTER I

# LITERATURE REVIEW

## *1. INTRODUCTION –INSULIN RECEPTOR*

Like many other polypeptide hormones insulin initiates its cellular action by binding to a specific hormone receptor on the plasma membrane of the cell. The coupling of insulin with its receptor triggers a wide spectrum of biological effects including enhanced transport of sugars and amino acids, stimulation of anabolic pathways, and promoting cellular growth by the initiation of gene transcription (1). Insulin is produced and secreted by the beta cells of the pancreas, when carbohydrate and protein are ingested (2). Insulin then exerts its effects on organs that express receptors that specifically recognize and bind the hormone. Mammalian cells are known to express 100 to 250, 000 receptors on their cell surface. The cells expressing the highest number of receptors ie. adipose, muscle and liver cells, are the most responsive to insulin stimulation (1,3). Therefore it follows that the most important tissues with respect to insulin action are the liver, skeletal muscle, and adipose tissue.

A significant body of knowledge regarding insulin action has been obtained since insulin receptors were first demonstrated in the early 1970's (4,5). Since then, the basic mechanism of receptor action; its specific recognition and binding of insulin, and its subsequent activation, by the autophosphorylation of its tyrosine residues, has been extensively described (1,3). However, the relationship between insulin-stimulated activation of its receptor and the myriad of cellular processes that follow have not fully been elucidated. Insulin signaling after receptor activation involves a protein phosphorylation/dephophorylation cascade and the generation of second messengers, some of which are unidentified (6).

### *1.1 Structure of the Insulin Receptor*

The insulin receptor is a glycoprotein made up of two polypeptides linked together by disulfide bonds to form a heterotetrameric complex. The first polypeptide $\alpha$, was purified (7) and was found to migrate around 135 Kilo Daltons (KDa) using soduim dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The second polypeptide $\beta$, was identified a few years later (8), to have an apparent molecular weight of 95 KDa. The native receptor has a stoichiometry of $\alpha_2\beta_2$ and an estimated molecular mass of 350 KDa (1). The $\alpha$-subunits are located entirely on the outside of the plasma membrane and are joined by disulfide bonds to the $\beta$-subunits which transverse the membrane. A model describing the structural topology of the receptor subunits was determined from experiments where the receptor was iodinated on tyrosine residues within intact plasma membrane vesicles and then specifically immunoprecipitated by using antireceptor antibodies (9). The $\alpha$-subunit was iodinated in right side-out vesicles only, whereas the $\beta$-subunit was labeled in both right side-out and inside-out vesicles. This model was confirmed when the insulin receptor gene was cloned and its amino acid sequence deduced (10). Within the $\beta$-subunit only, a single 23 amino acid hydrophobic segment was identified as the transmembrane domain of the receptor (10,11).

The susceptibility of the intact receptor to reductants such as dithiothreitol (DTT) and 2-mercaptoethanol, suggests that it is held together by disulphide bonds (12,13). The intact receptor was subjected to reducing conditions, followed by the alkylation of the free cysteine residues, and the separation of the resulting subunits by SDS-PAGE. Investigators demonstrated that a total of five disulphide bonds were responsible for holding together the complex oligomeric structure (14). Three of these disulphide bonds are between the homologous subunits ($\alpha$ -$\alpha$) or ($\beta$-$\beta$) of the receptor complex and are readily assessible to reduction. In contrast the two disulphide bonds between the $\alpha$ and the $\beta$ subunits and are virtually impossible to reduce without denaturing the protein (13). An additional structural characteristic of the insulin receptor is that the extracellular domains of both subunits are covalently attached to carbohydrate groups that bind the protein moiety through asparagine or serine residues (10,11). The formation of disulphide bonds between the subunits and the addition of sugars occur during the translocation of the nascent protein from its site of synthesis to its final location in the plasma membrane (15).

### *1.2 Ligand Binding*

The binding of insulin to its receptor is a specific function of the $\alpha$-subunit. The insulin binding domain of the $\alpha$-subunit contained was determined using labeling studies where radiolabeled insulin was bound and covalently attached to the receptor with a crosslinking agent, disuccinyl suberate (16,17). This amino-specific crosslinking agent covalently linked [$^{125}$-I]-insulin to the $\alpha$-subunit whereas crosslinking to the $\beta$-subunit was much weaker or did not occur at all (18,19). Addition of nanomolar quantities of unlabelled insulin was able to completely inhibit binding of the labeled hormone, indicating the high affinity and specificity of the insulin receptor for its ligand. The amino acid sequence deduced from the cloned gene offered additional evidence of an insulin binding domain in the $\alpha$-subunit. This region contains a high proportion of cysteine residues, which are homologously spaced with the cysteine residues in the extracelluar domains of other peptide hormones receptors, such as the insulin-like growth factor receptor and the epidermal growth factor receptor (10). The network of disulphide bridges between the cysteine residues of the $\alpha$-subunit confer a rigid and compact structure to the subunit and produce a ligand binding domain that is highly specific for insulin (1).

Morphological studies using fluorescent- and radio-labeled hormone have localized the insulin receptor not only to the plasma membrane but to intracellular membranes such as the nuclear membrane, lysosomal membranes, the endoplasmic reticulum, and the Golgi apparatus (20-22). Although the presence of insulin receptor in the latter two compartments may simply reflect the intracellular pathway of newly synthesized receptors to the plasma membrane (23), the physiological relevance of insulin binding sites at the nuclear membrane, has not yet been determined. The presence of nuclear insulin binding sites suggests that insulin may be involved in nuclear processes. Such processes include insulin-mediated regulation of the mRNA synthesis of certain genes (24-26).

The binding of insulin to its receptor is assayed by competitive binding assays using $^{125}$I-labeled insulin and receptor either within intact membranes or in solubilized and partially purified form (27). Typically $^{125}$I-labeled insulin is incubated with cells in the presence of varying amounts on unlabeled hormone at optimum conditions. After steady state binding has been attained, the radiolabeled hormone bound to membrane or to isolated receptors is then separated from the free hormone by high speed centrifugation through polyethylene glycol (27). Non-specific binding and cellular uptake of the radiolabel by non-receptor mediated process such as adsorption of insulin to the cell membrane is determined by the addition of a molar excess of unlabelled hormone to the reaction mixture. This large amount of unlabeled insulin effectively inhibits radiolabeled insulin binding to the receptor by saturating insulin-binding sites. Non specific binding is then subtracted from total binding to measure the amount of specific binding (27).

Kinetic analysis of insulin binding data employs Scatchard plots, where the concentration of bound hormone divided by free hormone is plotted on the vertical axis and the concentration of bound hormone is plotted on the abscissa (28). An advantage of the Scatchard plot is that it allows the estimation of the affinity constant of the receptor and the receptor concentration from the slope and the x-intercept respectively (28). This is most accurately determined when the plot is linear indicating homogeneity of reactants and a monovalent receptor or non-cooperation between multiple sites of a polyvalent receptor. However, this is not usually the case as many Scatchard plots of hormone-receptor binding, including insulin-receptor binding, are curvilinear. This non-linearity is generally attributed to heterogeneity of the receptor sites, each having different affinity constants, or to the presence of cooperativity between allosteric sites. Scatchard analysis of insulin binding data usually reveals concave upward plots, suggesting more than one independent binding site, with different receptor affinity and receptor concentration, or a single class or receptors with negative cooperative interactions (28-30). Negative cooperativity is ligand-induced dissociation of bound insulin. Evidence from recent studies suggests that the insulin receptor has a maximum of three binding sites for insulin and that each becomes occupied at high insulin concentrations (30). The estimation of the number of binding sites from a Scatchard plot by extrapolation of the curve to the x-intercept is usually subject to bias and results in a high amount of variability (31). In addition, the estimation of the affinity constants for receptors with negative cooperative interactions is also subject to variability since these quantities are based on estimated receptor concentration values (31). Other methods are being considered to estimate receptor affinity, such as the determination of the amount of unlabelled receptor required to inhibit 50 % of labeled hormone binding (31).

### *1.3 Insulin Receptor Tyrosine Kinase*

The β-subunit of the insulin receptor functions to anchor the protein in the plasma membrane and to activate a signal upon insulin binding. Kasuaga et al., (32) first demonstrated that insulin binding to the receptor induces the phosphorylation of the receptor. The phosphorylation was catalyzed by a kinase located within the receptor. It was subsequently found that tyrosine phosphorylation of the β-subunit further activates this kinase (33). In highly purified insulin receptor preparations, the addition of insulin stimulates the phosphorylation of tyrosine residues on the protein (33-37). However, in intact cells insulin stimulates the phosphorylation of not only tyrosine residues but of serine and threonine residues as well (33,35,38). This led investigators to believe that the insulin receptor is a tyrosine kinase, however, the series of proteins that it activates by tyrosine phosphorylation may include enzymes with serine or threonine kinase activity (1,3).

The mechanism by which insulin binding induces the autophosphorylation of its receptor has not yet been elucidated. Evidence suggests that insulin binding promotes aggregation of the extracellular domains of the receptor (39). This aggregation may cause a structural change resulting in kinase activation (40-42).

Characteristics of insulin receptor tyrosine kinase have been determined using antibodies directed against the insulin receptor (43,44), by site-directed receptor mutation analysis (45,46) and by making comparisons for homology with sequences of other growth factor receptors including the insulin-like growth factor receptor and the epidermal growth factor receptor (47). Insulin activates the autophosphorylation of its receptor three-fold in freshly isolated hepatocytes, with half maximum effects at approximately 2 nM (48,49). The kinase is specific for ATP and has a requirement for divalent cations such as $Mg^{++}$ or $Mn^{++}$ (34). Upon exposure of intact cells to insulin increases in phosphorylation are seen within 1 minute, and maximum phosphorylation occurs within 20 minutes. Phosphorylation is restricted to the $\beta$-subunit (32,33). Following insulin stimulation, the effect of insulin on receptor phosphorylation appears to be time-dependent. In the basal state, the receptor contains only phospho-serine and -threonine residues. However, with insulin exposure, a rapid rise in tyrosine phosphorylation occurs that reaches a steady state within 20 seconds. This is followed by a slower increase in phosphoserine content of the receptor, that reaches a maximum in about 10 minutes (35,48). The immediate phosphorylation of tyrosine residues upon insulin stimulation supports the concept that tyrosine phosphorylation activates the receptor tyrosine kinase. Using anti-phosphotyrosine antibodies to precipitate phosphotyrosine-containing receptors, investigators have demonstrated that the phosphorylation of tyrosine residues is inhibited when insulin receptors contain phosphoserine residues (35). This suggests that serine phosphorylation serves as the means to regulate insulin receptor kinase activity, by feedback inhibition.

Inactivation of insulin receptor tyrosine kinase activity by site-directed mutagenesis of specific tyrosine residues (45,46,49,50), or by anti-receptor monoclonal antibody binding to the ATP binding domain, compromises biological responses to insulin (45,46,49-51). However, there is some evidence that some aspects of insulin action may be independent of receptor tyrosine kinase activity. Recently, studies have shown that certain monoclonal antibodies may bind to the insulin receptor and mimic certain insulin-induced cellular responses without stimulating the receptor's tyrosine kinase activity (52,53). Binding of these antibodies to the insulin receptor may induce a conformational change similar to that caused by autophosphorylation. This conformational change may stimulate a membrane bound signal transducer, possibly a GTP-binding protein that may mediate insulin cellular responses (54). Evidence for this hypothesis has been provided by several studies in which pertussis toxin, an inactivator of GTP-binding -proteins, was shown to block certain actions of insulin (55). Therefore, it may be possible that insulin receptor autophosphorylation produces receptor kinase activation as well as the non-covalent activation of specific membrane-bound proteins.

### *1.4 Insulin Signal Transduction*

Insulin binding to the receptors triggers the activation of a complex of intracellular proteins that mediate insulin action. As insulin action diverse and seemingly unrelated events ranging from glucose transport to DNA transcription, mediators of insulin action are likely to be equally diverse. The first step in transducing the signal once insulin binds to its receptor is the tyrosine phosphorylation of cellular proteins. Proteins phosphorylated on tyrosine in response to insulin stimulation have been difficult to identify. Less than 0.1 % of proteins are phosphorylated on tyrosine residues, therefore putative substrates of the insulin receptor are present in very low amounts (56). To remedy this detection problem researchers now use antibodies directed against phosphotyrosine to selectively immunoprecipitate and isolate these proteins from [$^{32}$P]-phosphate labeled, insulin-treated cells (57). Following insulin stimulation, a major protein species that is recognized by a phosphotyrosine antibody appears on SDS-PAGE. This protein referred to as insulin receptor substrate-1 (IRS-1), migrates around 185 KDa on SDS-PAGE gels (57), and is the most characterized of all insulin stimulated tyrosine phosphorylated proteins (6). There is substantial evidence suggesting that this protein is important in insulin action. Following insulin stimulation, the events of autophosphorylation and IRS-1 tyrosine phosphorylation appear to be closely tied (57). Cells expressing kinase defective receptors show a significant decrease in IRS-1 phosphorylation. (6). A putative IRS-1 binding domain on the insulin receptor has been identified. Cells expressing insulin receptor having mutations in this region are unable to phosphorylate IRS-1, and also do not exhibit some insulin-stimulated biological effects, including the activation of glycogen synthase, amino acid uptake, and thymidine incorporation into DNA (58). These receptors exhibited normal insulin binding and insulin stimulated autophosphorylation, suggesting that mutations made in the IRS-1 binding region affected subsequent steps in insulin action. The gene encoding IRS-1 has been cloned and sequenced, and the protein has had its amino acid profile analyzed (59). Its insulin receptor binding domain recognizes phosphorylated $Tyr_{960}$ of the insulin receptor. IRS-1 becomes phophorylated at eight tyrosine and at about 30 serine/threonine residues upon insulin stimulation (57,60). In the basal state IRS-1 is serine phosphorylated, upon insulin stimulation tyrosine phosphorylation and increased serine phosphorylation occurs (57,60). Two proteins with high homology to IRS-1 have been identified. Insulin receptor substrate-2 (IRS-2) and IRS-1 share extensive homology (61). Differences between the two proteins may be found in their cellular distribution and function (6). Another protein that becomes rapidly tyrosine phosphorylated upon insulin stimulation is Shc, a 60 KDa protein (61). Members of the IRS family are distinguished by multiple Tyr-Met-Xaa-Met motifs (YMXM), that become phophorylated on the tyrosine residues upon insulin stimulation (60). A phosphorylated tyrosine within the YMXM sequence allows the motif to be recognized by intracellular enzymes that are activated by IRS proteins (62). The $Y^{(P)}$MXM recognition domain has been termed an SH2 domain because it is homologous to a region of the src oncogene product (62). IRS proteins interact with proteins containing SH2 domains including phosphatidylinositol 3'kinase (P-I-3'kinase) (60), Growth factor receptor bound-2 (GRB-2) an upstream regulator of p21$^{ras}$ (63), and two tyrosine phosphatases, SHPTP1C (64), and SHPTP2 (65). After IRS activation, insulin signaling diverges into distinct and possibly independent path, each directed by the action of SH2 containing proteins, P-I-3'K, GRB2, or SHPTP2 (6). P-I-3'kinase catalyzes the phosphorylation of phosphatidylinositol (PI), phosphatidylinositol-4 –phosphate (PI-4-P), and phosphatidylinositol-4,5,-bisphosphate (PI-4,5-$P_2$) on the 3' position to form PI-3-P, PI-3,4-$P_2$, and PI-3,4,5-$P_3$, respectively (66). Phosphatidylinositol compounds are activators of protein kinase C which lies downstream of insulin action (6,67). P-I-3'K is responsible for mediating the activation of ribosomal S6 kinase a (p70$^{s6K}$), a protein that is translocated to the nucleus. The pathway directed by P-I-3'K may play a role in mitogenic signaling by insulin (26).

Insulin stimulation results in the activation of the ras -mitogen activated protein kinase (MAP-K) pathway, a pathway distinct from the signaling mediated by P-I-3'K. p21$^{ras}$ is a GTP-binding protein which is activated by GRB-2 via a protein named Son of Sevenless (SOS) (26,68). Both SHPTP2 and the GTP-bound form of p21$^{ras}$ stimulates the serine/threonine Raf kinase. Raf-1 kinase phosphorylates and activates MAP kinase-kinase, referred to as MEK. MEK activation leads to the phosphorylation of the MAP kinase family (6,69). The MAP kinases are serine-threonine kinases that can translocate into the nucleus and phosphorylate nuclear proteins such as p90 ribosomal-S6-kinase (70) and glycogen synthase kinase (GSK-3) (71). The culmination of the insulin signal in the nucleus via MAP-kinase suggests that there is a connection between MAP kinase cascade and insulin regulation of gene expression.

Evidence to date suggests that insulin intracellular signaling is mediated through IRS proteins. By using IRS proteins, as intermediates, the insulin receptor signal can be amplified. It has been suggested however, that some cellular responses to insulin may not depend upon IRS activation. Disruption of IRS gene blunts but does not ameliorate mitogenic signaling by insulin (6,72).

## *2 INSULIN ACTION*

Insulin functions to maintain plasma glucose homeostasis, to stimulate anabolic pathways and to promote cell growth and differentiation (73). Its most critical role is to strictly regulate circulating glucose levels by stimulating glucose uptake into the liver and peripheral tissues (74). Nevertheless, insulin also controls enzymes responsible for glucose metabolism, glycogen and lipid storage, amino acid transport, protein synthesis and mitogenesis (73). Insulin modulates the activity of enzymes at the level of gene expression, and by post-translational modification of mature proteins. In the first case, insulin has been demonstrated to influences the levels of several proteins expressed in the cell by regulating their rate of transcription (26). In the second, insulin produces acute changes in enzyme activity by covalent modification of the phosphorylation state of the enzymes (75). Numerous hormones counterregulate insulin action in the hypoglycemic state and during physiological stress, including glucagon, adrenaline, cortisol, and growth hormone (76). However insulin is generally the dominant glucoregulatory homone under physiological conditions, which emphasizes the magnitude of insulin's effect on biological systems (74).

### *2.1 Glucose Uptake into Muscle and Fat*

Insulin secretion from the pancreatic beta-cell is stimulated by the increase in plasma glucose levels that follows a meal (2). The hormone acts chiefly on muscle and adipose tissue cells, and stimulates glucose uptake from the blood. Glucose transporter proteins are mobilized from intracellular compartments to the plasma membrane (77). In fat cells, in the absence of insulin, less than 10% of glucose transporters are associated with the membrane, instead they are located within intracellular vesicles. Within 10 minutes of insulin exposure, about 50 to 60% of the transporters are in the plasma membrane, exposed to the external milieu (78,79). Translocation may be initiated by changes in phosphorylation state of the glucose transporters. Insulin or phorbyl ester-stimulated activation of protein kinase C has been demonstrated to result in glucose uptake (80). Protein kinase C phosphorylates glucose transporter proteins *in vitro*, but not *in vivo* (80). MAP kinase has also been suggested to be involved in insulin signaling of the glucose transport system (81).

Several isoenzymes of glucose transporter (GLUT-1, GLUT-2, GLUT-3, GLUT-4, and GLUT-5, and SGLT-1) have been identified, which differ in insulin responsiveness and tissue expression (82,83). GLUT-1 and GLUT-3 isoforms are found in plasma membranes of most cell types including muscle, liver and adipose and are involved in glucose transport under basal conditions (82,83). In adipose and muscle cells, insulin stimulation accelerates basal glucose transport 20 - 30 fold with the translocation of GLUT-4 from an intracellular pool to the plasma membrane. GLUT-4 is chiefly responsible for the majority of insulin-stimulated of glucose uptake in these cells (84).

GLUT-2 is uniquely expressed in gastrointestinal epithelial cells, in liver and beta cells of islets. It is the predominant glucose transporter isoform found in the liver and is responsible for both glucose uptake from the circulation and glucose release to the circulation from the liver. SGLT-1 is the glucose transporter found in the basolateral membrane of the gut epithelial cells. GLUT-2 and SGLT-1 activities do not appear to be regulated by insulin (85).

### *2.2 Glucose Metabolism*

Following a meal, insulin suppresses endogenous glucose production in the liver (75). It inhibits the key enzymes of gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase, and glucose-6-phosphatase and prevents glycogen breakdown by inhibiting glucogen phosphorylase (75,86). Insulin also promotes glucose utilization, predominantly in muscle, by stimulating enzymes for glycolysis including pyruvate kinase, 6-phosphofructo 1-kinase, glucokinase, and glyceraldehyde-3-phophate dehydrogenase (GAPDH) (75). These enzymes are subject to both short term regulation by insulin via covalent modification and long term regulation by insulin via gene expression. By suppressing hepatic glucose production and by increasing glucose utilization, in muscle and adipose tissue, insulin lowers plasma glucose concentration.

### *2.3 Glycogen Storage*

Following meal ingestion, insulin directs the flow of glucose into glycogen and inhibits glycogen breakdown. Several hours after a meal, the insulin counterregulatory hormones glucagon and epinephrine, promote glycogen degradation in an effort to raise plasma glucose concentrations (75). Glycogen is synthesized in muscle, liver and fat cells by glycogen synthase. The enzyme is activated by insulin in muscle and fat cells, and to a lesser extent in hepatocytes (86). Following exposure to insulin, the phosphorylation state of glycogen synthase decreases (87). Dephosphorylation of glycogen synthase is believed to be catalyzed by an insulin responsive serine/threonine phosphatase (88). Insulin stimulates dephosphorylation of glycogen synthase by an another mechanism. In rat adipocytes, the increase in intracellular glucose-6-phosphate concentration resulting from insulin-stimulated glucose transport, increases glycogen synthase phosphatase activity (89). Glycogen degradation to glucose-1-phosphate is catalyzed by glycogen phosphorylase. In the organs with substantial glycogen stores, such as muscle, liver and kidney, glycogen phosphorylase is activated upon its phosphorylation by a specific phosphorylase kinase. Insulin prevents the action of this kinase by phosphorylase kinase dephosphorylation (90). In liver and kidney cells, glucose-1-phosphate is converted to glucose-6-phosphate, which is then dephosphorylated to glucose by glucose-6-phosphatase before it is released into the circulation (90). Although glycogenolysis does occur in muscle cells, these cells do not express significant levels of glucose-6-phosphatase, and therefore do not contribute much to the blood glucose concentration (91).

### *2.4 Lipid Synthesis and Storage*

Following a high carbohydrate meal, insulin stimulates fatty acid synthesis in the liver and adipose tissue and suppresses lipolysis in adipose tissue, thus promoting lipid storage (74). Key enzymes involved in fatty acid metabolism are regulated by insulin. Pyruvate dehydrogenase catalyzes the irreversible conversion of pyruvate to acetyl CoA, thus acetyl CoA cannot be used for net glucose synthesis (3). Acetyl CoA produced when there are adequate carbohydrate reserves is shunted into fatty acid synthesis. In the mitochondria of fat cells, insulin causes an acute increase in pyruvate dehydrogenase activity (92). Insulin-induced dephosphorylation of pyruvate dehydrogense, catalyzed by an insulin responsive phosphatase, increases pyruvate dehydrogenase activity (93).

Conversion of acetyl CoA into malonyl CoA, catalyzed by acetyl CoA carboxylase, is the rate limiting step in long chain fatty acid synthesis. Insulin exposure causes phosphorylation of some (and the simultaneous dephosphorylation of other) residues within acetyl CoA carboxylase, which results in an increase in enzyme activity (94-96). Insulin stimulates the activity of ATP citrate lyase, an enzyme that provides NADPH used in fatty acid production. Insulin exposure causes the phosphorylation of ATP citrate lyase on specific serine residues (97). In the fed state, insulin prevents fatty acid breakdown by inhibiting the action of hormone-sensitive lipase. This enzyme catalyzes the breakdown of adipose triglycerides into free fatty acids and glycerol, which are then transported to the liver (98). Insulin blocks the phosphorylation of hormone-sensitive lipase (99). Key enzymes involved in fatty acid synthesis including fatty acid synthase, malic enzyme, and acetyl CoA carboxylase are subjected to acute regulation by insulin at the level of gene expression (26).

### *2.5 Protein Metabolism*

Following a meal, insulin promotes anabolism of protein by increasing the transport of most amino acids into muscle (74). In the normal physiological state, insulin promotes net protein synthesis and inhibits proteolysis (74) Protein is catabolized to synthesize glucose during physiological stressful conditions such as starvation, infection and cancer. Under these conditions insulin action is countered by cortisol (76).

## *2.6 Gene Expression*

The amount of a specific protein in a cell is in part determined by its rate of synthesis, a process that begins at gene transcription, and ends after messenger RNA (mRNA) has been translated and the product has been processed into the mature protein. Cytoplasmic mRNA levels are influenced by mRNA synthesis, by the rate of mRNA transport from the nucleus and by mRNA stability in the cytoplasm (24-26). Insulin has been demonstated to affect the levels of cytoplasmic mRNA found in eukaryotic cells, and many of these changes appear to be mediated at the level of gene transcription (24-26). Many studies have examined the effects of insulin on the rates of expression of various genes *in vivo* by nutritional and hormonal manipulation of insulin levels. The expression of several insulin-responsive genes including those for pyruvate kinase, glucokinase, and lipogenic enzymes are increased by a high carbohydrate diet, and are decreased by fasting and the insulin-deficient diabetic state (100,101). In this experimental system however, these differences are hard to interpret and cannot be attributed solely as insulin-mediated. Carbohydrate metabolism may play an independent or permissive role in the regulation of some insulin-responsive genes. For example stimulation of liver pyruvate kinase gene expression can occur if hepatocytes are treated with low concentrations of fructose, in the absence of an increase in insulin concentration (178). It has been suggested that glucose metabolites such as glucose-6-phosphate and pyruvate, rather than glucose, may be critical components of the carbohydrate-induced gene expression (179,180). In this instance, insulin may play a permissive role in carbohydrate-mediated gene expression as insulin acts to stimulate the glycolytic process (181). The effect of insulin on the regulation of genes can be complex when studied in the whole animal model. Insulin-induced hypoglycemia causes the secretion of counterregulatory hormones which may complicate insulin action (26).

Transcriptional regulation by insulin is demonstrated more directly using tissue culture lines, primary cell culture, or tissue explants that have been treated with insulin under conditions where the cellular environment is controlled. Gene transciption rates in response to insulin exposure can be measured by nuclear run-on assays that determines the incorporation of labeled nucleotides into newly synthesized mRNA (24).

The expression of many of the key enzymes in glycolysis is increased upon insulin stimulation. In the liver, the mRNA levels of pyruvate kinase, 6-phosphofructo-1-kinase and glucokinase are reduced in fasting and diabetes and restored to normal levels by refeeding and insulin administration, respectively (102-104). Nuclear run-on experiments using liver nuclei from diabetic animals have demonstrated a twenty-fold increase in the glucokinase gene transcription rate after insulin administration of insulin (105). A seven-fold increase was observed in the pyruvate kinase gene transcription rate (106). In contrast, insulin suppresses the synthesis of key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and fructose 1,6-bisphosphatase (26). In rat hepatoma cells previously treated with a cAMP analog to increase the rate of PEPCK synthesis, physiological concentrations of insulin (1 μM) abolished the stimulatory effect of cAMP, by reducing mRNA synthesis to basal levels (107). This inhibitory effect was acute ($t_{1/2}$= 20 min), appeared to be mediated by the specific binding of insulin to its receptor and was reversible upon removal of the hormone (107). Insulin suppression of PEPCK gene expression occurred without prior stimulation by cAMP, indicating that the two regulators functioned via independent mechanisms. Further studies using gene transfer, have identified putative sequences in the promotor region of the PEPCK gene which are required for negative regulation by insulin. This PEPCK-insulin responsive element (IRE) when fused to the chloroamphenicol-acetyl transferase (CAT) promotorless gene construct conferred negative control of CAT expression in response to insulin in transfected cells (108).

Insulin also regulates the expression of a number of proteins not directly involved in glycolysis and gluconeogenesis. The list includes hormones such as prolactin, thyroglobulin, and growth hormone, enzymes such as pancreatic amylase, glutamine synthetase, and tyrosine aminotransferase and protooncogenes such as c-fos (24-26).

Insulin is able to illicit both acute (observed within min to one hour) and long term (observed within several hours to days) changes on the rate of transcription of gene (25). In intact animals, changes in ob (109), and L-type pyruvate kinase (110) gene expression were observed within 2-4 hours of insulin stimulation but changes in FAS gene expression were not observed until after 16 h following insulin stimulation (111). Using cells in culture, transcription regulation was observed within 5 min for c-fos (112) and within 30 min for PEPCK (113) genes but was not observed for FAS (114) and prolactin (115) genes until 16 hours and 24 hours, respectively.

Specific sequences within the promoter region of genes are necessary for gene transcription to be responsive to insulin. These sequences are recognized by, and bound by, transcription factors, nuclear proteins that bind to the promoter region and enhance or repress the rate of transcription by interacting with the initiation machinery (24-26). Insulin responsive elements (IRE) have been identified within the promoters of several genes (26). Among known IREs no consensus sequence has been identified to date (26). However close similarities have been observed between the IRE for GAPDH, ME and FAS genes (116,117). A comparison of the IRE within PEPCK gene with those of other insulin-regulated genes demonstrates some similarities to amylase and insulin like growth factor binding protein 1 genes (118). An approach used to identification an IRE uses a promoter construct containing the putative insulin-responsive element fused to the promoter of a non-responsive gene. The transcription rate of this chimeric gene is then determined upon exposure to insulin (24). Once the IRE is identified, double-stranded oligonucleotides corresponding to its sequence may be used to screen cDNA libraries constructed in prokaryotic expression vectors for clones expressing proteins that specifically bind to the oligonucleotide probes (119). This approach is used to identify transcription factors which bind to IREs. DNA-protein binding is analyzed using DNAase 1 footprinting, methylation interference, and gel retardation assays. Transcription factor-binding to the IRE within GAPDH (120), and ME (116) genes has been demonstrated. It has not been determined the transcription factors that bind to homologous IRE motifs found within different genes are common proteins.

Within the promoter regions of some insulin-responsive genes, DNA sequences have been identified that confer responsiveness to glucose. Carbohydrate responsive elements (ChoRE), have been studied in the genes coding for liver pyruvate kinase and $S_{14}$ (179). Homology exists between insulin response elements (core motif CGCCTC) (116) and carbohydrate response elements (core motif CACGTG) (181), which suggests that common DNA-binding factor(s) may control expression of genes the harbor these sequences. Such a system would allow for synergistic interaction between metabolic and hormonal signals (181).

Insulin affects other nuclear processes such as nuclear transport, and mRNA stability (24,25). In order to stimulate nuclear transport of mRNA, insulin is thought to increase the activity of nuclear membrane triphosphatase (NTPase), an enzyme that catalyzes the energy-driven export of metabolites through pores in the nuclear membrane (121,122) and of nucleolin, a nuclear lamin (123). Insulin decreases the phosphorylation state of both proteins thereby stimulating enzyme activity. This effect of insulin may have been mediated by specific binding of insulin to binding sites at the nuclear membrane (121).

## *3 INSULIN ACTION AT THE NUCLEUS*

### *3.1 Mediators of Insulin Action at the Cell Nucleus*

The effects of insulin in the nucleus are likely to be mediated by nuclear DNA-binding proteins that are activated by insulin signaling. The protein phosphorylation/dephosphorylation cascade triggered by the insulin receptor tyrosine kinase upon insulin binding include proteins that may have a mitogenic action. One such group of proteins is the MAP kinase family. The MAP kinases appear to be central to the action of a number of growth factors and are activated by insulin (124,125). MAP kinase has been demonstrated to translocate to the nucleus following stimulation with serum or with growth factors (126). Potential nuclear substrates of MAP kinase include p90 ribosomal-S6-kinase and GSK-3 (70,71). Signaling mediated through the MAP kinase pathway is thought to be responsible for insulin-regulation of c-fos gene transcription as well as other transcription factors in the activator protein-1 (AP-1) family (26). Inhibition of $p21^{ras}$ (127), or expression of kinase defective MAP kinase blocks c-fos gene expression (128). Evidence suggests that a connection between MAP kinase and a transcription factor associated with the c-fos serum response element may exist. The binding affinity of $p62^{TCF}$ to a serum response element within the c-fos promoter has been demonstrated to increase in insulin-treated cells (129). Increasing the phosphorylation of $p62^{TCF}$ increases its binding to the serum response element within c-fos gene (129), and MAP kinase has been demonstrated to phosphorylate $p62^{TCF}$ *in vitro* (130). Transcription factors, c-fos and c-jun, have been demonstrated to become phosphorylated upon insulin stimulation, possibly via MAP kinase (131). These transcription factors recognize AP-1 sites within collagenase and albumin genes, thus MAP kinase-mediated AP-1 phosphorylation may be involved in insulin regulation of gene trancription (132).

Insulin-regulation of gene expression may be through the activation of P-I-3'K (26,133). Inactivation of P-I-3'K by wortmannin blocks the effect of insulin on hexokinase (133) and PEPCK gene expression (134). The direction of the signal after PI 3'K activation is not clear. $p70^{s6K}$ a downstream molecule of the PI 3'-K signal appears to be involved in insulin-induced hexokinase (133), but not (135) insulin regulation of PEPCK gene expression.

Evidence suggests that multiple signaling pathways mediate insulin regulation of gene expression. The time course of insulin action is diverse, observed within min for PEPCK and c-fos gene induction or slow in onset (hours or days for FAS) (26,111). Deletion of the IRS-1 gene by targeted disruption is not able to block insulin stimulation of mitogenesis (6,72). Mutant insulin receptors with reduced autophosphorylation activity but normal ability to activate IRS-1, show marked impairment of glycogen synthesis (50). Insulin-induced expression of egr-1, a member of the activator protein-1 family, does not require insulin receptor substrate-1 phosphorylation (136). Inhibition of $p21^{ras}$ activation reveals that insulin-induced stimulation of glycogen synthase is ras-and MAP-kinase independent (137). Insulin-induced c-fos stimulation of gene expression has been demonstrated to require MAP-kinase but not IRS-1 and PI 3'K activation (136).

Although activation of some intracellular mediators of insulin signaling are not essential for insulin regulation of gene expression, insulin receptor ligand binding, autophosphorylation and internalization may be indispensable. Insulin fails to stimulate

DNA synthesis upon tyrosine kinase inactivation of the insulin receptor and impaired internalization of the insulin-receptor complex (138). Taken together this evidence suggests the possibility of an alternate pathway of insulin signaling to the nucleus; one that bypasses activation of IRS-1.

### *3.2 Insulin Accumulation in the Nuclear Membrane*

Nuclear processes influenced by insulin may be affected by direct interaction of insulin receptor with the nucleus. Studies using ferritin- or gold-labeled insulin complexed to its receptor have provided evidence of insulin accumulation in the nucleus (139,140). This insulin-stimulated accumulation of labeled particles appeared to be dose-dependent and saturable, suggesting that these effect are mediated by a nuclear envelope insulin receptor (139,140). Smith and Jarrett, have demonstrated that, at high insulin concentrations, insulin uptake into cell nuclei is non-saturable suggesting that insulin may be internalized via nuclear pores (139). It is thought that insulin binds to receptors, which translocate to the nucleus and mediate the nuclear insulin response, similar to insulin signaling at the cell membrane. Insulin receptors have been recovered in isolated rat liver nuclear membranes of animals in the fed state (141-143). However insulin-binding sites were observed in nuclear membranes isolated from liver of fasted rats in one study (144) suggesting that receptors could be found in the nucleus in the basal state. Two studies have tracked insulin receptor accumulation in cultured cells after stimulation with insulin. In one study using hepatocytes (145), insulin receptor was covalently crosslinked to insulin to its receptor in intact cells at $4^0$C. Following incubation at $37^0$C, cell nuclei were isolated and insulin receptors quantified by autoradiography. Insulin receptor was not present in nuclei maintained at $4^0$C, presumably because insulin receptor internalization was impaired. Incubation of cells at $37^0$C for 30 min resulted in the accumulation of labeled insulin receptors. In a recent study, immunodetection methods were used to track the appearance of insulin receptor in nuclei isolated of cultured adipocytes. Insulin receptor levels in isolated nuclei increased within five min following stimulation with insulin (146). The nuclear insulin receptor has been demonstrated to be phosphorylated on the β-subunit and to exhibit tyrosine kinase activity towards exogenous substrates (141,146).

Insulin receptor is internalized following ligand stimulation via a process termed endocytosis. Following internalization, insulin receptor-ligand complexes are localized to endosomes and then lysosomes where insulin dissociates from its receptor and is degraded. Insulin receptor then recycles back to the plasma membrane where it is reused (147). A nuclear localization signal has been identified by computer analysis within the amino acid sequence of the insulin receptor, which may function to target the receptor to the cell nucleus (148). Nuclear translocations of the receptors for other growth factors including growth hormone receptor (149) and epidermal growth factor (150) have been demonstrated.

Insulin accumulation in the nucleus may in part be non-receptor mediated. Cultured hepatoma cells treated with trypsin to abolish cell surface insulin binding and insulin-stimulated tyrosine kinase activity, were able to internalize insulin and accumulate the hormone in the nucleus (151). In addition, insulin accumulation in the nucleus was demonstrated to affect the transcription of insulin-responsive genes c-fos and g33. It must be noted that supraphysiological concentrations of insulin (100 nM) were used in order to stimulate non-receptor-mediated internalization of the insulin, therefore these observations may not have physiological significance.

## *4 INSULIN ACTION AT THE NUCLEUS: FACTORS AFFECTING INSULIN RECEPTOR FUNCTION*

### *4.1 Dietary Fat Alters the Structure of the Nuclear Envelope*

Changing the levels of specific fatty acids in the diet can alter plasma membrane lipid composition. Increasing the ratio of dietary polyunsaturated to saturated fat (P/S) increases the total polyunsaturated fatty acid content of adipocyte plasma membrane including the content of 18:2(ω-6) and 20:4(ω-6) in all membrane phospholipid classes (152). The nuclear envelope is also responsive to dietary fatty acid manipulation. A similar relationship between dietary P/S ratio and lipid composition is seen in the hepatocyte nucleus. Rats and mice fed a high P/S diet have higher levels of 18:2(ω-6) in phosphotidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) and higher levels of 20:4(ω-6) in PC, compared to animals fed a low P/S diet (153-155). The ratio of ω-6 to ω-3 polyunsaturated fatty acids (PUFA) in the nuclear envelope can also be manipulated by diet. Substituting increasing levels of 20:5(ω-3) and 22:6(ω-3) for 18:2(ω-6) in a low P/S diet resulted in a displacement of ω-3 for long-chain (LC) PUFA in the nuclear membrane (156). Nuclear membrane fluidity and/or protein interactions and subsequently membrane protein function may be influenced by the increased incorporation of dietary polyunsaturated fatty acids in the nuclear membrane.

### *4.2 Dietary Fat, Disease and the Nuclear Envelope*

Obesity and diabetes are characterized by abnormalities in lipid metabolism. When compared to normal animals, obese and diabetic animals show marked changes in membrane fatty acid composition. For example, feeding high fat diets to lean and obese animals results in lower levels of 20:4(ω-6) in membrane phospholipids in obese animals (157). Both streptozotocin-induced diabetes and genetically spontaneous diabetes have been associated with a decrease in the amount of LCPUFA, a decrease correctable by insulin therapy (158-160). The decrease in membrane LCPUFA characteristic of both obesity and diabetes suggests that the process of desaturation-elongation of the essential fatty acids is impaired. These conversions are catalyzed by delta-6 desaturase, responsible for the formation of 20:3(ω-6) from 18:2(ω-6), and delta-5 desaturase, responsible for the formation of 20:4(ω-6) from 20:3(ω-6). In genetically obese mice and rats, a lower delta-5 desaturase activity has been observed (157,161,162). Streptozotocin-induced diabetes has been shown to be associated with decreased activity of microsomal delta-9- and delta-6 desaturase in the liver (159). Evidence suggests that abnormal insulin production, as seen in diabetes, and in obesity-induced insulin resistance causes impaired desaturation/elongation of membrane lipid. This evidence indicates the desaturase enzymes are insulin-dependent (163).

Obesity (143,154) and diabetes induce changes in nuclear membrane composition. Nuclear membrane isolated from the liver of rats having genetically spontaneous diabetes was associated with an increase in 18:2(ω-6) and a decrease in 20:4(ω-6) in PC as diabetes progressed, suggesting a decrease in desaturase activity (164). Decreased levels of 20:4(ω-6) observed in nuclear membrane phospholipid of streptozotocin-induced diabetic animals were normalized by islet transplantation (163). Streptozotocin-induced diabetes resulted in a lower content of 20:4(ω-6) in PE in the rat liver nuclear envelope (154). Dietary fat was able to abrogate the pertubations on the nuclear envelope fatty acid composition induced by diabetes and obesity. High PUFA diets raised the levels of 20:4(ω-6) in liver nuclear membrane PC of animals with spontaneous diabetes (165).

### *4.3 Relationship Between Dietary Fat, Disease and Insulin Receptor Function in the Nucleus.*

The insulin receptor is a membrane bound protein and therefore is influenced by factors that determine membrane constituents and physical environment. Changes in membrane lipid composition induced by diet fat and by obesity and diabetes do have an impact on the function of the insulin receptor (152). A positive relationship was observed between membrane PUFA content upon high-fat, high P/S feeding and insulin receptor ligand binding. In rat adipocytes, a high P/S diet induced an increase in insulin receptor function as assessed by insulin binding, which was coupled to increases in insulin-stimulated functions within the cell, including glucose transport, glucose oxidation, and lipid synthesis (166). A similar relationship between nuclear membrane composition and insulin function in the nucleus may exist. The physical properties of the nuclear membrane may influence the insulin receptor trafficking and translocation to the nucleus. In the spontaneous diabetic BB rat, insulin binding to hepatocyte nuclear receptors decreases as diabetes becomes more pronounced (164). Scatchard analysis reveals that a high and a low affinity binding site exist in the nucleus of control animals, but only a single class of binding sites is observed in diabetic animals. Insulin binding levels are increased in both diabetic and control animals by a high P/S diet compared to a low P/S or chow diet. However high PUFA feeding only partially restored the reduced levels of insulin binding is observed in diabetic animals (164). In diabetes induced by streptozotocin, nuclear insulin receptors isolated from liver had higher insulin binding capacity compared to control animal (163). Only one class of binding site was seen in diabetic animals, whereas high and low affinity binding was observed in control animals. Improved insulin binding in the nuclei of diabetic animals may be associated with an upregulation of insulin receptor protein levels induced by low circulating insulin (167). Insulin receptors may become more available or up-regulated in insulin deficiency and conversely downregulated in response to hyperinsulinemic states such as obesity (167). Low PUFA diets decreased and high PUFA diets increased insulin binding to nuclei in both control and diabetic animals compared to chow feeding. Insulin resistance occurs when an increased amount of insulin is required to elicit a given response. Obesity, a disease characterized by insulin resistance, was associated with reduced insulin binding to liver nuclei. In the genetically spontaneous obese mouse (ob/ob), obesity decreased insulin binding to hepatocyte nuclei compared to controls (143). In this study (143), high PUFA feeding increased insulin binding in both lean and obese animals. These findings suggest that increasing the polyunsaturated fatty acid content of nuclear membrane phospholipids by high PUFA feeding is associated with improved insulin binding capacity in the nucleus in both normal and disease states. Increases in receptor binding induced by nuclear membrane lipid environment may affect the insulin receptor function in the nucleus by improving the activity of insulin receptor tyrosine kinase. Both dietary changes (168) and membrane lipid modulation *in vitro* (169) of the plasma membrane environment was demonstrated to have an effect on the function of insulin receptor tyrosine kinase within the plasma membrane. Thus it is feasible to expect a similar relationship between lipid environment and receptor function in the liver nucleus.

### *4.4 Effects of Dietary Fat and Obesity on Insulin Action at the Cell Nucleus*

Insulin regulation of nuclear processes is affected by nutrition and by disease states such as diabetes and obesity. PUFA feeding suppress the transcription of insulin-responsive genes including FAS (170,171), stearoyl-CoA desaturase (172) ME and l-type pyruvate kinase (158). PUFA-responsive cis-acting elements have been identified in the FAS gene (173). Hyperinsulinemia and altered lipid composition in membrane lipids may be responsible for an overexpression of FAS and other lipogenic enzymes in obese animals compared to lean controls (174-176). Thus, in contrast to the stimulatory effect of insulin on lipogenic gene expression, PUFA seem to have an inhibitory effect. In obese animals fed high fat diets, a high P/S diet lowered mRNA level of FAS and ME compared to a low P/S diet (177). This evidence suggests that despite hyperinsulinemia, PUFA feeding is able to repress lipogenic gene overexpression in obese animals. As PUFA are able to enhance insulin receptor function in the nucleus, it was expected that PUFA feeding would potentiate insulin-induced overexpression of lipogenic gene expression observed in obese animals compared to lean animals. Thus the ability for PUFA to enhance insulin receptor function in the liver nucleus appears to be independent of PUFA-induced suppression of gene expression.

## 5 RATIONALE AND OBJECTIVES

The liver is a site for numerous metabolic processes including glucose oxidation, glycogen synthesis, gluconeogenesis, lipogenesis, and protein synthesis. Insulin plays a critical role in the regulation of these processes. Insulin receptors have been detected at the cell nucleus however the mechanism of nuclear trafficking of the insulin receptor and its function in the nucleus is not known. Ligand-stimulated receptor translocation to the nucleus may be a mechanism by which insulin regulates the expression of various metabolic genes. Insulin receptor function in the plasma membrane is altered by changes in membrane lipid composition induced by polyunsaturated fat feeding or by disease state such as diabetes and obesity. Insulin receptor function in the nucleus may be similarly modulated by changes in nuclear membrane composition. Altered insulin receptor function in the cell nucleus may cause impair regulation of gene expression by insulin, which may be part of the etiology of obesity induced-insulin resistance.

**The objectives of this thesis research is to demonstrate that:**

1. Insulin stimulation induces the translocation of the insulin receptor to the hepatocyte nucleus.
2. Newly translocated insulin receptor in the hepatocyte nucleus has tyrosine kinase activity and is able to phosphorylate nuclear proteins.

3. Insulin receptor translocation to the nucleus will cause changes in nuclear protein phosphorylation and insulin-responsive gene expression.
4. Endogenous substrates in the nucleus can be identified by direct incubation of cell nuclei with insulin receptor *in vitro*.
5. Insulin receptor translocation to the nucleus is influenced in the obese state.

## *6 HYPOTHESES*

**It is specifically hypothesized that:**

> **Hypothesis 1:** Insulin receptor levels in isolated cell nuclei of hepatocytes taken from fasted animals will increase following an oral glucose meal and an increase in serum insulin concentration.

Chapter 2 investigates the effect of an oral glucose meal on serum glucose, serum insulin concentrations and the presence of insulin binding sites on the hepatocyte nucleus of mice fasted for 12 hours. Chapter 4 examines the effect of oral glucose meal on serum concentrations of glucose and insulin and on insulin receptor levels in isolated nuclei taken from mice fasted for 24 hours. Insulin receptor levels in the nucleus were assayed using immunoassay.

> **Hypothesis 2:** Insulin receptor levels in isolated cell nuclei of hepatocytes taken from fasted animals are decreased in obese animals compared to lean animals following an oral glucose meal.

Chapter 3 investigates the effect of obesity on internalization of insulin receptor from the surface of the cell and translocation of insulin receptor to the hepatocyte nucleus following an oral glucose meal to fasted lean and obese animals.

**Hypothesis 3:** Insulin receptor translocation to the nucleus following an oral glucose meal is reflected in an increase in nuclear insulin receptor β-subunit autophosphorylation.

Chapter 4 describes the tyrosine phosphorylation state of the β-subunit of newly translocated insulin receptor at various time periods following oral glucose meal in mice fasted for 24 hours.

**Hypothesis 4:** Insulin receptor translocation to the nucleus following an oral glucose meal is coincident with increases in tyrosine phosphorylation of nuclear proteins.

Chapter 4 determines the tyrosine phosphorylation state of nuclear proteins at various time periods following an oral glucose meal in mice fasted for 24 hours.

**Hypothesis 5:** Insulin receptor translocation to the nucleus following an oral glucose meal is coincident with increases in expression of insulin-responsive genes including malic enzyme, fatty acid synthase and glyceraldehyde 3-

phosphate dehydrogenase.

Chapter 4 investigates the effect of an oral glucose meal on mRNA levels of insulin responsive genes involved in glucose and lipid metabolism, including malic enzyme, fatty acid synthase and glyceraldehyde 3-phosphate dehydrogenase detected in liver of mice fasted for 24 hours

**Hypothesis 6:** Insulin receptor translocation to the nucleus following an oral glucose meal is coincident with increases in transcription factor binding to an insulin responsive sequence.

Chapter 5 examines oral glucose induced changes in association of transcription factors to insulin responsive sequences within the promoter regions of insulin-responsive genes.

**Hypothesis 7:** Direct incubation of insulin receptor with cell nuclei isolated from hepatocytes taken from fasted animals will increase phosphorylation of nuclear proteins.

Chapter 5 investigates the effect of incubation of purified insulin receptor on the phosphorylation state of nuclear proteins isolated from fasted animals.

**Hypothesis 8:** Direct incubation of insulin receptor with cell nuclei isolated from hepatocytes taken from fasted animals will increase the binding of transcription factors to an insulin-responsive sequence

Chapter 5 examines changes in association of transcription factors to insulin-responsive sequences within the promoter regions of insulin-responsive genes induced by purified insulin receptor.

## 7 REFERENCES

1. Zick Y. The insulin receptor: Structure and function. Crit Rev Biochem Mol Biol, 1989;24(3):217-269.

2. Flatt, P.R., Barne, H.L.R., Snibier, O., and Swanson-Flatt, S.K. Direct and indirect actions of nutrients in the regulation of insulin secretion from the pancreatic beta cells (1991) Proc. Nutr. Soc.,50(3):559-566.

3. Denton, R.M. Early events in insulin actions. *In*, Advances in Cyclic Nucleotide and Protein Phosphorylation Research, vol 20, Greengard P. and Robison, G.A., eds. Raven Press, New York, 1986,293-341.

4. Cuatrecasas P. Insulin-receptor interactions in adipose tissue cells: direct measurement and properties. Proc Natl Acad Sci USA, 1971;68:1264-1268.

5. Freychet P, Roth J, and Neville DM jr. Insulin receptors in the liver: specific binding of 125I-insulin to the plasma membrane and its relation to insulin bioactivity. Proc Natl Acad Sci USA, 1971;68:1833-1837.

6. Myers, M.G., and White, M.F. Insulin signal transduction and the IRE proteins. (1996) Ann. Rev. Pharmacol. Toxicol.,36:615-658.

7. Jacobs S, Shechter Y, Bissell K and Cuatrecasas P. Purification and properties of insulin receptors from rat liver membranes. Biochem Biophys Res Commun, 1977;77:981-988.

8. Fugita-Yamaguchi Y, Choi S, Sakamoto Y, and Itakura K. Purification of insulin receptor with full binding activity. J Biol Chem, 1983;258:5045-5049.

9. Hedo J and Simpson IA. Internalization of insulin receptors in the isolated rat adipose cell. Demonstration of the vectorial disposition of receptor subunits. J Biol Chem, 1984;259:11083-11089.

10. Ebina Y, Ellis L, Jarnagin K, Edery M, Graf L, Clauser E, Ou JH, Masiarz F, Kan Y, Goldfine ID, Roth RA, and Rutter WJ. The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling. Cell, 1985;40:747-758.

11. Ullrich A, Bell JR, Chen EY, Herrera R, Petruzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M, Mason A, Seeburg PH, Grunfeld C, Rosen OM, and Ramachandran J. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature (London), 1985;313:756-761.

12. Boni-Schnetzler M, Scott W, Waugh SM, DiBella E, and Pilch PF. The insulin receptor. Structural basis for high affinity ligand binding. J Biol Chem, 1987;262(17):8395-8401.

13. Massague J and Czech MP. Role of disulfides in the subunit structure of the insulin receptor. Reduction of class I disulfides does not impair transmembrane signalling. J Biol Chem, 1982;257:6729-6738.

14. Finn FM, Ridge KD, and Hofmann K. Labile sisulfide bonds in human placental insulin receptor. Proc Natl Acad Sci USA, 1990;87:419-423.

15. Olson TS, Bamberger MJ and Lane MD. Post translational changes in tertiary and quaternary structure of the insulin proreceptor. Correlation with acquisition of function. J Biol Chem, 1988;263:7342-7351.

16. Pilch PF, and Czech MP. The subunit structure of the high affinity insulin receptor. Evidence for a disulfide-linked receptor complex in fat cell and liver plasma membranes. J Biol Chem, 1980;255:1722-1731.

17. Pilch PF, and Czech MP. Interaction of cross linking agents with the insulin effector system of isolated fat cells. Covalent linkage of 125I-insulin to a plasma membrane receptor protein of 140,000 daltons. J Biol Chem, 1979;254:3375-

18. Massague J, Pilch PF, Czech MP. A unique proteolytic cleavage site on the subunit of the insulin receptor. J Biol Chem, 1981;256:3182-

19. Jacob S, Hazum E, Schechter Y, and Cuatrecasas P. Insulin receptor: covalent labeling and identification of subunits. Proc Natl Acad Sci USA, 1979;76:4918-

20. Gordon P, Carpentier JL, Freychet P, Le Cam A, and Orci L. Intracellular translocation of iodine-125-labelled insulin: direct demonstration in isolated hepatocytes. Science, 1978;200:782-786.

21. Goldfine ID, Smith GJ, Wong K-Y, and Jones AL. Cellular uptake and nuclear binding of insulin in human cultured lymphocytes: evidence for potential intracellular site of insulin action. Proc Natl Acad Sci USA, 1977;74:1368-1372.

22. Carpentier JL. The cell biology of the insulin receptor. Diabetologia, 1989;32:627-635.

23. Knutson VP. Cellular trafficking and processing of the insulin receptor. FASEB J, 1991;5:2130-2138.

24. Meisler, M.H., and Howard, G. Effects of insulin on gene transcription. (1989) Annu. Rev. Physiol.,51:710-714.

25. Castano JG. Regulation of gene expression by insulin. Adv Enz Regul, 1991;31:185-194.

26. O'Brien, R.M., and Granner, D.K. Regulation of gene expression by insulin. (1996) Physiol. Rev.,76(4):1109-1161.

27. Pederson, O. Studies of insulin receptor binding and insulin action in humans. (1994) Dan. Med. Bull.,31(1):1-32.

28. Scatchard G. The attractions of proteins for small molecules and ions. Ann N Y Acad Sci, 1949;51-660-672.

29. De Meyts P, Bianco AR, and Roth J. Site-site interactinos amoung insulin receptors. Characterization of the negative cooperativity. J Biol Chem, 1976;215:1877-1888.

30. De Meyts, P. The structural basis of insulin and insulin-like growth factor-1 receptor binding and negative co-operativity, and the relevance to mitogenic versus metabolic signaling. (1994) Diabetol.,(suppl2):s135-s148.

31. Rodbard D. Mathematics of hormone-receptor interaction. In: Receptors for reproductive hormones. O'Mally BW and Means AR (Eds). Plenum Publishing Co. New York, 1973:289-326.

32. Kasuga M, Karlsson FA, and Kahn CR. Insulin stimulates the phosphorylation of the 95000-dalton subunit of its own receptor. Science, 1982;215:185-187.

33. Kasuga M, Zick Y, Blithe DL, Crettaz M, and Kahn CR. Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell free system. Nature, 1982;298:667-669.

34. Kahn CR, White MF, Grigorescu F, Takayama S, Haring HU, and Crettaz M. The insulin receptor protein kinase . In: Molecular Basis of Insulin Action, Czech MP. Ed, Plenum Press, New York, 1985,67-94.

35. Pang DT, Sharma BR, Shafer JA, White MF, and Kahn CR. Predominance of tyrosine phosphorylation of insulin receptors during the initial response to intact cells to insulin. J Biol Chem, 1985;260:7131-7136.

36. Kasuga M, Fujita-Yamaguchi Y, Blithe DL, White MF, and Kahn CR. Characterization of the insulin receptor kinase purified from human placental membranes. J Biol Chem, 1983;258:10973-

37. Petruzzeli LM, Herrera R, and Rosen OM. Insulin receptor is an insulin dependant tyrosine protein kinase: copurification of insulin binding activity and protein kinase activity to homogeneity from human placenta. Proc Natl Acad Sci USA, 1984;81:3227

38. Kasuga M, Zick Y, Blithe DL, Karlsson FA, Haring HU, and Kahn CR. Insulin stimulation of the beta subunit of the insulin receptor. J Biol Chem, 1982;257:9891-

39. Ellis L, Tavare JM, and Levine BA. Insulin receptor tyrosine kinase structure and function. Biochem Soc Trans, 1991;19(2):426-432.

40. Kahn CR, Baird KL, Jarrett DB, and Flier PS. Direct demonstration that receptor crosslinking or aggregatin is important in insulin action. Proc Natl Acad Sci USA, 1978;75:4209-

41. Schechter Y, Hernaez L, Schlessinger J, and Cuatrecasas P. Local aggregation of hormone-receptor compleses is required for activation by epidermal growth factor. Nature (London), 1979;278:835-

42. Boni-Schnetzler M, Rubin JB, and Pilch PF. Structural requirements for the transmembrane activation of the insulin receptor kinase. J Biol Chem, 1986;261:15,281-

43. Taylor, R., Soos, M.A., Argyraki, M., and Siddle, K. Insulin-like and insulin-inhibitory effects of monoclonal antibodies for different epitopes on the human insulin receptor. (1987) Biochem. J.,242:123-129.

44. Morgan DO, and Roth RA. Acute insulin action requires insulin receptor kinase activity: introduction of an inhibitory monoclonal antibody into mammalian cells blocks the rapid effects of insulin. Proc Natl Acad Sci USA, 1987;84:41-45.

45. Chou, C.K., Dull, T.J., Russell, D.S., Gherzi, R., Lebwohl, D., Ullrich, A., and Rosen, O.M. Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate post-receptor effects of insulin. (1987) J. Biol. Chem.,262:1842-1847.

46. Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C.S., Siddle, K., Pierce, S.B., Roth, R.A., and Rutter, W.J. Replacement of lysine residues 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. (1987) Proc. Natl. Acad. Sci. USA,704-708.

47. Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussins, L., Liao, Y-C. Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M., and Ramachandran, J. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. (1985) Nature,313:756-761.

48. Ballotti, R., Kawalski, A., White, M.F., Le Marchand-Brustel, Y., and Van Obberghen, E. Insulin stimulates tyrosine phophorylation of its receptor β-subunit in intact rat hepatocytes. (1987) Biochem. J.,241:99-104.

49. Rafealoff, R., Maddux, B.A., Brunetti, A., Spraccia, P., Sung, C.K., Patel, R., Hawley, D.M., and Goldfine, I.D. Transmembrane signaling by insulin via an insulin receptor mutated at tyrosines 1158, 1162 and 1163. (1991) Biochem. Biophys. Res. Comm.,179:912-918.

50. Wilden, P.A., Backer, J.M., Kahn, C.R., Cahill, D.A., Schroeder, G.J., and White, C.F. Characterization of the insulin receptor with an *in vitro* mutation at tyrosine

1146: evidence for separate insulin receptor signals regulating cellular metabolism and growth. (1990) Proc. Natl. Acad. Sci. USA,87:3358-3362.

51. White, M.F., Shoelson, S.E., Keutmann, H., and Kahn, C.R. A cascade of tyrosine autophophorylation in the β-subunit activates the phosphorylation of the insulin receptor. (1988) J. Biol. Chem.,263:2969-2978.

52. Forsayeth JR, Caro JF, Sinha MK, Maddux BA, and Goldfine ID. Monoclonal antibodies to the human insulin receptor that activate glucose transport but not insulin receptor kinase activity. Proc Natl Acad Sci USA, 1987;84:3448-3453.

53. Hawley DM, Maddux BA, Patel RG, Wong K-Y, Mamula PW, Firestone GL, Burnetti A, Verspohl E, and Goldfine ID. Insulin receptor monoclonal antibodies that mimic insulin action without activating tyrosine kinase. J Biol Chem, 1989;2438-2444.

54. Saltiel AR. Second messangers of insulin action. Diabetes Care, 1990;13(3):244-256.

55. Goren JJ, Northrup JK, and Hollenberg MD. Action of insulin modulated by pertussis toxin in rat adipocytes. Can J Physiol Pharmacol, 1985;63:1017-1022.

56. Cooper JA, Bowen-Pope PF, Raines E, Ross R, and Hunter T. Similar effects of platelet-derived growth factor and epidermal growth factor on the phosphorylation of tyrosine in cellular proteins. Cell, 1982;31:263-.

57. White, MF, Maron R, and Kahn CR. Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. Nature, 1985;318:183-186.

58. Wilden P.A., Kahn, C.R., Siddle, K., and White, M.F. Insulin receptor kinase domain autophosphorylation regulates receptor enzymatic function. (1992) J. Biol. Chem., 1992;267:16660-16668.

59. Sun X-J, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, and White MF. The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature (London), 1991;352:73-77.

60. Sun, X-J., Crimmons, D.L., Myers, M.G. Jr., Miralpeix, M., and White, M.F. Pleiotropic insulin signals are engaged by multisite-phosphorylation of IRS-1. (1993) Mol. Cell. Biol.,13:7418-7428.

61. Sun, X-J., Wang, L.M., Zhang, Y., Yenush, L.P., Myers, M.G., et al. Role of IRE-2 in insulin and cytokine signaling. (1995) Nature,377:173-177.

62. Koch, C.A., Anderson, D.J., Morah, M.F., Ellis, C., Pawson, T. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. (1991) Science,252:668-674.

63. Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., et al. Guanine-nucleotide releasing factor mSOS1 binds to GRB2 and links receptor tyrosine kinases to ras signaling. (1993) Nature,363:85-88.

64. Uchida, T., Motozaki, T., Noguchi, T., Yamoa, T., Horita, K., Suzuki, T., Fugioka, Y., Sakamoto, C., and Kasuga, M. Insulin stimulates the phosphorylation of $Tyr^{538}$ and the catalytic activity of PTP1C, a protein tyrosine phosphatase with src homology-2 domains. (1994) J. Biol. Chem.,269(16):12220-12228.

65. Staubs, P.A, Reichart, D.R., Saltiel, A.R., Milarski, K.L., Maegawa, L.I., Berhanu, P., Olefsky, J.M., and Seely, B.L. Localization of the insulin receptor sites for the SH2 domain proteins p85, Syp, and GAP. (1994) J. Biol. Chem.,269(44):27186-27192.

66. Whitman, M., Downes, C.P., Keller, M., Ketter, T., and Cantley, L. Type 1 phosphatidylinositol kinase makes a novel inositol phospholipid, phophatidylinositol-3-phosphate. (1988) Nature,332:644-646.

67. Nakanishi, H., Brewer, K.A., Exton, J.H. Activation of the zeta isozyme of protein kinase by phosphatidylinositol 3,4,5-trisphosphate. (1993) J. Biol. Chem.,268:13-16.

68. Eagan, S.E., Giddins, B.W., Brooks, M.W., Buday, L., Sizeland, A.M., and Weinberg, R.A. Association of SOS-ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. (1993) Nature,363:45-51.

69. Roberts, T.M. A signal chain of events. (1992) Nature,360:534-35.

70. Sturgill, T.W., Ray, L.B., Erikson, E., and Maller, J.L. Insulin-stimulated MAP-2 kinase phophorylates and activates ribosomal protein S6 kinase II. (1988) Nature Lond.,334:715-718.

71. Sutherland, C., and Cohen, P. The α-isoform of glycogen synthase kinase-3 from rabbit skeletal muscle is inactivated by p70S6 kinase or MAP kinase-activated protein kinase-1 *in vitro*. (1994) FEBS Lett.,338:37-42.

72. Araki, E., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, B. III, Johnson, R.J., and Kahn, C.R. Alternative pathway of insulin signaling in mice with targeted disruption of the IRS-1 gene. (1994) Nature,372:186-190.

73. Lonnroth, P. Regulation of insulin action at the cellular level. (1991) J. Int. Med.,229(Suppl.2):23-29.

74. Cryer PE. Regulation of glucose metabolism in man. (1991) J. Int. Med.,229(Suppl. 2):31-39.

75. Pilkis SJ, and Granner DK. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. (1991) Ann. Rev. Physiol.,54:885-909.

76. Lager I. The insulin-antagonistic effect of the counterregulatory hormones. . (1991) J. Int. Med.,229(Suppl. 2):41-47.

77. Suzuki K, and Kono T. Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. (1980) Proc. Natl. Acad. Sci USA,77:2542-2545.

78. Kono T. Insulin-dependant apparent tranlocation of glucose tranport activity. In: Molecular Basis of Insulin Action. Czech MP, ed. Plenum Press, New York, pp423-431.

79. Simpson IA, and Cushman SW. Hexose transport regulation by insulin in the isolated rat adipose cell. In: Molecular Basis of Insulin Action, Czech MP, ed. Plenum Press, New York, pp399-422.

80. Kitagawa K, Nishino H, and Iwashima A. Tumor promoter-stimulated translocation of glucose transport system in mouse embryo fibroblast Swiss 3T3 cell. (1985) Biochem. Biophys. Res. Commun.,128:1303-1309.

81. Inoue G, Kuzuya H, Hayashi T, Okamoto M, Yoshimasa Y, Kosaki A, Kono S, Okamoto M, Maeda i, Kubota M, et al. Effects of ML-9 on insulin stimulation of glucose transnport in 3T3-L1 adipocytes. (1993) J. Biol. Chem.,268:5272-5278.

82. Bell GI, Kayano T, Buse JB et al. Molecular biology of mammalian glucose transporters. (1990) Diabetes Care,13:198-208.

83. Pessin JP, and Bell GI. Mammalian facilitative glucose transporter family: Structure and molecular regulation. (1992) Ann. Rev. Physiol.,54:911-930.

84. Hirshman MF, Goodyear LJ, Wardzala LJ, Horton ED, and Horton ES. Identification of an intracellular pool of glucose transporters from basal and insulin-stimulated rat skeletal muscle. (1990) J. Biol. Chem.,265:987-991

85. Thorens B, Cheng Z-Q, Brown D, and Lodish HF. Liver glucose transporter: a basolateral protein in hepatocytes and intestine and kidney cells. (1990) Am. J. Physiol.,259:C279-C285.

86. Larner J. Insulin and the stimulation of glycogen synthesis. The road from glycogen structure to glycogen synthase to cyclic AMP-dependant protein kinase to insulin mediators. (1990) Adv. Enzymol.:173-231.

87. Parker PJ, Caudwell B, and Cohen P. Glycogen synthase from rabbit skeletal muscle: Effect of insulin on the state of phosphorylation of the seven phosphoserine residues *in vivo*. (1983) Eur. J. Biochem,130:227-234.

88. Lawrence JC Jr. Signal transduction and protein phosphorylation in the regulation of cellular metabolism by insulin. (1992) Annu. Rev. Physiol.,54:177-193.

89. Lawrence JC Jr, and James C. Activation of glycogen synthase by insulin in rat adipocytes: Evidence of hormonal stimulation of multisite dephosphorylation by glucose transport-dependant and independent pathways. (1984) J. Biol. Chem.,259:7975-7982.

90. Hems DA, and Whitton PD. Control of hepatic glycogenolysis. (1980) Physiol. Rev.,60:1-50

91. Lackmer R, Challis A, West D, et al. A problem in the radiochemical assay of glucose-6-phosphate in muscle. (1984) Biochem. J.,218:649-651.

92. Stansbie D, Brownsey RW, Crettaz M, and Denton RM. Acute effects *in vivo* of anti-insulin serum on rates of fatty acid synthesis and activities of acetyl coenzyme A carboxylase and pyruvate dehydrogenase in liver and epidedymal adipose tissue in fed rats. Biochem J, 1976;160:413-416.

93. Denton RM, Midgley PJW, Rutter GA, Thomas AP, and McCormack JG. Studies into the mechanism whereby insulin activates pyruvate dehydrogenase complex in adipose tissue. Ann NY Acad Sci, 1989;573:285-295.

94. Bothwick AC, Edgell NJ, and Denton RM. Protein-serine kinase from rat epidedymal adipose tissue which phosphorylates and activates acetyl-CoA carboxylase. Biochem J, 1990;270:795-801.

95. Haystead TA, Campbell DG, and Hardie DG. Analysis of sites phosphorylated on acetyl-CoA carboxylase in responce to insulin in isolated adipocytes. Comparison with sites phosphorylated by casein kinase-2 and the calmodulin-dependent multiprotein kinase. Eur J Biochem, 1988;175:347-354.

96. Mabrouk GM, Helmy IM, Thampy K, and Wakil SJ. Acute hormonal control of acetyl-CoA carboxylase. J Biol Chem, 1990;265:6330-6338.

97. Yu K-T, Benjamin WB, Ramakrishna S, Khalaf N, and Czech MP. An insulin-sensitive cytosolic protein kinase accounts for the regulation of ATP citrate-lyase phosphorylation. Biochem J, 1990;268:539-545.

98. Yeaman SJ. Hormone-sensitive lipase - a multipurpose enzyme in lipid metabolism. Biochim Biophys Acta, 1990;1052:128-132.

99. Stralfros P and Honnor RC. Insulin-induced dephophorylation of hormone-sensitive lipase. Correlation with lipolysis and cAMP-dependent protein kinase activity. Eur J Biochem, 1989;182:379-385.

100. Iritani, N., Hosomi, H., Fukuda, H., and Ikeda, H. A review: nutritional and hormonal regulation of lipogenic gene expression in rat liver. (1992) Eur. J. Biochem.,205:433-442.

101.Coupe, C., Perdereau, D., Ferre, P., Hitier, Y., Narkewicz, M., and Girard, J. (1990) Am. J. Physiol.,258,E126-133.

102.Magnuson MA, Andreone TL, Printz RL, Koch S, and Granner DK. The rat glucokinase gene: Structure and regulation by insulin. Proc Natl Acad Sci USA, 1989;86:4838-4842.

103.Gehnrich SC, Gekakis N, and Sul HS. Liver (L-type) phosphofructokinase mRNA. J Biol Chem, 1988;263:11755-11759.

104.Pool GP, Postle AD, and Bloxham DP. The induction of synthesis of L-type pyruvate kinase in cultured rat hepatocytes. Biochem J, 1982;204:81-87.

105.Iynedjian PB, Gjinovci A, and Renold AE. Stimulation by insulin of glucokinase gene transcription in liver of diabetic rats. J Biol Chem, 1988;263:740-744.

106.Noguchi T, Inoue H, and Tanaka T. Transcriptional and post-transcriptional regulation of L-type pyruvate kinase in diabetic rat liver by insulin and dietary fructose. J Biol Chem, 1985;260:14393-14397.

107.Granner DK, Kazuyuki S, Adreone T, and Beale E. Insulin regulates expression of the phosphoenolpyruvate carboxykinase gene. Rec Prog Horm Res, 1986;42:111-141.

108.Magnuson MA, Quinn PG, and Granner DK. Multihormonal regulation of phosphoenolpyruvate carboxykinase-chloramphenicol acetyltransferase fusion genes: Insulin's effects oppose those of cAMP and dexamethasone. J Biol Chem, 1987;262:14917-14920.

109.Saladin, R., De Vos, P., Guerre-Millo, M., Leturque, A., Girard, J., Staels, B., and Auwerx, J. Transient increase in obese gene expression after food intake or insulin administration. (1995) Nature,377:527-529.

110.Iritani, N., Fukuda, H., Tada, K., Itoh, A., and Noguchi, T. Diet differentially regulates glucokinase and L-type pyruvate kinase gene expression in rat liver. (1995) J. Nutr.,125:2945-2952.

111.Soncini, M., Yet, S-F., Moon, Y., Chun, J-Y., and Sul, H.S. Hormonal and nutritional control of the fatty acid synthase promoter in transgenic mice. (1995) J. Biol. Chem.,270(51):30339-30343.

112.Messina, J.L. Insulin's regulation of c-fos gene transcription in hepatoma cells. (1990) J. Biol. Chem.,265(20):11700-11705.

113.Granner, D., Andreone, T., Sasaki, K., and Beale, E. Inhibition of transcription of the phosphoenolpyruvate carboxykinase gene by insulin. (1983) Nature,305:549-551.

114.Paulauskis, J.D., and Sul, H.S. Cloning and expression of mouse fatty acid synthase and other specific mRNAs. Developmental and hormonal regulation in 3T3-L1 cells. (1988) J. Biol. Chem.,263:7049-7054.

115.Stanley, F. Stimulation of prolactin expression by insulin. (1988) J. Biol. Chem.,263:13444-13448.

116.Garcia-Jimenez, C., Benito, B., Jolin, T., and Santisteban, P. Insulin regulation of malic enzyme gene expression in rat liver: evidence for nuclear proteins that bind to two putative insulin responsive sequences. (1994) Mol. Endocrinol.,8:1361-1369.

117.Moustaid, N., Beyer, R.S., and Sul, H.S. Identification of an insulin response element in the fatty acid synthase promoter. (1994) J. Biol. Chem.,269(8):5629-5634

118.O'Brien, R.M., and Granner, D.K. Regulation of gene expression by insulin. (1991) Biochem. J.,278:609-619.

119.Singh, H., LeBowitz, J.H., Baldwin, A.S. Jr., and Sharp, P.A. Molecular cloning of an enhancer binding protein: Isolation by screening of an expression library with a recognition site DNA. (1988) Cell,52:415-.

120.Nasrin, N., Ercolani, L., Denaro, M., Kong, X.F., Kang, I., and Alexander, M. An insulin responsive element in the glyceraldehyde-3-phosphate dehydrogenase gene binds a nuclear protein induced by insulin in cultured cells and by nutritional manipulatons *in vivo*. (1990) Proc. Natl. Acad. Sci. USA,87:5273-5277.

121.Goldfine ID, Purrello F, Vigneri R, and Clawson GA. Direct regulation of nuclear functions by insulin: relationship to mRNA metabolism. In: Molecular Basis of Insulin Action. Czech MP, ed. Plenum Press, New York, 1985:329-345.

122.Purrello, F., Burnham, D.B., and Goldfine, I.D. Insulin regulation of protein phosphorylation in isolated rat liver nuclear envelopes: Potential relationship to mRNA metabolism. (1983) Proc. Natl. Acad. Sci. USA,80:1189-1193.

123.Csermely, P., Schnaider, T., Cheatham, B., Olson, M.O.J., and Kahn, C.R. Insulin induces the phosphorylation of nucleolin. A possible mechanism of insulin-induced RNA efflux from nuclei. (1993) J. Biol. Chem.,268(13):9747-9752.

124.Ray, L.B., and Sturgill, T.W. Rapid stimulation by insulin of a serine/threonine in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 *in vitro*. (1987) Proc. Natl. Acad. Sci. USA,84:1502-1506

125.Rossomondo, A.J., Payne, D.M., Weber, M.K., and Sturgill, T.W., Evidence that pp42, a major tyrosine kinase target protein, is a mitogen activated serine/threonine protein kinase. (1989) Proc. Natl. Acad. Sci. USA, 86:6940-6943.

126.Chen, R-H., Sarnecki, C., and Blenis, J. Nuclear localization and regulation of erk- and ras-encoded protein kinases. (1992) Mol. Cell. Biol.,12:912-927.

127.Medema, R.H., Wubbolts, R., and Bos, J.L. Two dominant inhibitory mutants of $p21^{ras}$ interfere with insulin-induced gene expression. (1991) Mol. Cell. Biol.,11:5963-5967.

128.Westwick, J.K., Cox, A.D., Der, C.J., Cobb, M.H., Hibi, M., Karin, M., and Brenner, D.A. Oncogenic Ras activates c-Jun via a separate pathway from the activation of extracellular signal-regulated kinases. (1994) Proc Natl. Acad. Sci. USA,91:6030-6034.

129.Thompson, M.J., Roc, M.W., Malik, R.K., and Blackshear, P.J. Insulin and other growth factors induce binding of the ternery complex and a novel protein complex to the c-fos serum response element. (1994) J. Biol. Chem.,269:21127-21135.

130.Hipskind, R.A., Rao, V.N., Mueller, C.G.F., Reddy, E.S.P., and Nordheim, A. Ets-related protein Elk-1 is homologous to the c-fos regulatory factor $p62^{TCF}$. (1991) Nature Lond.,354:531-534.

131.Kim, S-J., and Kahn, C.R. Insulin stimulates phophorylation of c-Jun, c-fos and Fos-related proteins in cultured adipocytes. (1994) J. Biol. Chem.,269(16):11887-11892.

132.Hu, J., and Isom, H.C. Suppression of albumin enhancer activity by H-ras and AP-1 in hepatoma cell lines. (1994) Mol. Cell. Biol.,14:1531-1543.

133.Osawa, H., Sutherland, C., Robey, R.B., Printz, R.L., and Granner, D.K. Analysis of the signaling pathway involved in the regulation of hexokinase II gene transcription by insulin. (1996) J. Biol. Chem.,271(28):16690-16694.

134.Sutherland, C., O'Brien, R.M., and Granner, D.K. Phosphatidylinositol 3-kinase, but not p70/p85 ribosomal S6 protein kinase, is required for the regulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression by insulin. J. Biol. Chem.,270:15501-15506.

135.Chung, J., Kuo, C.J., Crabtree, G.R., and Blenis, J. Rapamycin-FKBP specifically blocks growth dependent activation of and signaling by the 70kd S6 protein kinases. (1992) Cell,69:1227-1236.

136.Harada, S., Smith, R.M., Smith, J.A., White, M.F., Jarett, L. Insulin-induced egr-1 and c-fos expression in 32D cells requires insulin receptor, Shc, and mitogen-activated protein kinase, but not insulin receptor substrate-1 and phosphatidylinositol 3-kinase activation. (1996) J. Biol. Chem.,271:30222-30226.

137.Sakaue, M., Bowtell, D., and Kasuga, M. A dominant-negative mutant of mSOS1 inhibits insuln-induced Ras activation and reveals ras-dependent and -independent insulin signaling pathways. (1995) Mol. Cell. Biol.,15(1):379-388.

138.Krook, A., Moller, D.E., Dib, K., and O'Rahilly, S. Two naturally occurring mutant insuln receptor phosphorylated IRS-1 but fail to mediate the biological effects of insulin. Evidence that IRS-1 phosphorylation is not sufficient for normal insulin action. (1996) J. Biol. Chem.,271:7134-7140.

139.Smith RM, and Jarett L. Ultrastructural evidence for the accumulation of insulin in nuclei of intact 3T3-L1 adipocytes by an insulin-receptor mediated process. Proc Natl Acad Sci USA, 1987;84:459-463.

140.Soler AP, Smith RM, and Jarett L. Insulin stimulates accumulation and efflux of macromolecules in isolated nuclei from H35 hepatoma cells. Diabetes, 1992;41:194-201.

141.Wong, K.Y., Hawley, D., Vigneri, R., and Goldfine, I.D. Comparison of solubilized and purified plasma membrane and nuclear insulin receptors. (1988) Biochem.,27(1):375-379.

142.Vigneri R, Goldfine ID, Wong KY, Smith GJ, and Pezzino V. The nuclear envelope. The major site of insulin binding in rat liver nuclei. (1978) J. Biol. Chem.,253:2098-2103.

143.Cheema SK, Venkatraman J, and Clandinin MT. Insulin binding to liver nuclei from lean and obese mice is altered by dietary fat. Biochim Biophys Acta, 1992;1117:37-41.

144.Horvat A. Insulin binding sites on rat liver nuclear membranes: Biochemical and immunofluorescent studies. J Cell Physiol, 1978;97:37-48.

145.Podlecki DA, Smith RM, Kao M, Tsai P, Huecksteadt T, Brandenburg D, Lasher RS, Jarett L, and Olefsky JM. Nuclear translocation of the insulin receptor. J Biol Chem, 1987;262:3362-3368.

146.Kim, S-J., and Kahn, C.R. Insulin induces rapid accumulation of insulin receptors and increases tyrosine kinase activity in the nucleus of cultured adipocytes. (1993) J. Cell. Physiol.,157:217-228.

147.Carpentier, J.L. Insulin receptor internalization: molecular mechanisms and physiopathological implications. (1994) Diabetol.,37(suppl2):s117-s124.

148.Radulescu, R.T. Insulin receptor α-subunit: A putative gene regulatory molecule. (1995) Med. Hypothesis,45:107-111.

149.Lobie, P.E., Wood, T.J.J., Chen, C.M., Waters, M.J., and Norstedt, G. Nuclear translocation of the growth hormone receptors. (1994) J. Biol. Chem.,269(50):31735-31746.

150.Jiang, L-W., and Schindler, M. Nucleocytoplasmic transport is enhanced concomitant with nuclear accumulation of epidermal growth factor (EGF) binding activity in both 3T3-1 and EGF receptor reconstituted NR-6 Fibroblasts. (1990) J.

Cell. Biol.,110:559-568.

151.Lin YJ, Harada S, Loten EG, Smith RM, and Jarett L. Direct stimulation of immediate-early genes by intranuclear insulin in trypsin-treated H35 hepatoma cells. Proc Natl Acad Sci USA, 1992;89:9691-9694.

152.Field CJ, Ryan EA, Thomson ABR, and Clandinin MT. Diet fat composition alters membrane phospholipid composition, insulin binding, and glucose metabolism in adipocytes from control and diabetic animals. J Biol Chem, 1990;265:11143-11150.

153.Clandinin MT, Cheema S, Field CJ, Garg ML, Venkatraman J, and Clandinin TR. Dietary fat: exogenous determination of membrane structure and cell function. FASEB J, 1991;5:2761-2769.

154.Kang J, Venkatraman JT, and Clandinin MT. Influence of dietary lipids on the binding of adenosine to rat liver nuclear envelope: Effect of diabetes. Diab Nutr Metab, 1992;5:167-174.

155.Venkatraman, J.T., Lefebvre, Y.A., and Clandinin, M.T. Diet fat alters the structure and function of the nuclear envelope: modulation of membrane fatty acid composition, NTPase activity, and binding of triiodothyronine. (1986) Biochem. Biophys. Res. Comm.,135(2):655-661.

156.Venkatraman JT, Toohey T, and Clandinin MT. Does a threshold for the effect of dietary omega-3 fatty acids on the fatty acid composition of nuclear envelope phospholipids exist? Lipids, 1992;27:94-97.

157.Clandinin, M.T., Cheema, S., Pehowich, D., and Field, C.J. Effect of polyunsaturated fatty acids in obese mice. (1996) Lipids,31:S13-S22.

158.Minnouni, V., Narce, M., and Poisson, J.P. Evidence for insulin-dependent hepatic microsomal gamma-linoleneic acid chain elongation in spontaneously diabetic Wistar BB rats. (1992) Biochim. Biophys. Acta,1133:187-192.

159.Faas, F.H., and Carter, W.J. Altered fatty acid desaturated and microsomal fatty acid composition in the streptozotocin diabetic rat. (1980) Lipids,15953-961.

160.Poisson, J.P. Essential fatty acid metabolism in diabetes. (1989) Nutrition,5:263-266.

161.Cunnane, S.C., Manker, M.S., and Horobin, D.F. Abnormal essential fatty acid composition of tissue lipids in genetically diabetic mice is partially correctable by dietary linoleic and gamma-linolenic acids. (1985) Br. J. Nutr.,53:441-448

162.Guesnet, P.H., Bouree, J.M., Guerre-Millo, M., Pascal, G., and Durand, G. Tissue phospholipid fatty acid composition in genetically lean (fa/-) or obese (fa/fa) Zucker female rats on the same diet. (1990) Lipids,25:517-522.

163.Cheema, S.K., Rajotte, R.V., and Clandinin, M.T. Effect of diet fat on liver nuclei membrane phospholipid fatty acid composition and insulin binding after islet transplantation. (1996) Biochim. Biophys. Acta, submitted.

164.Cheema, S.K., and Clandinin, M.T. Diet and diabetes induced change in insulin binding to the nuclear membrane in spontaneously diabetic rats in association with change in fatty acid composition of phosphatidylinositol. Biochim. Biophys. Acta, submitted.

165.Cheema, S.K., and Clandinin, M.T. Effect of dietary polyunsaturated fat on gene expression in liver. *In* Advances in Polyunsaturated Fatty Acid Research, p.189-192. Elsevier. Amsterdam/New York (1993).

166.Field, C.J., Toyomizu, M., and Clandinin, M.T. Relationship between diet fat, plasma membrane lipid composition, and insulin-stimulated functions in isolated adipocytes. *In* Biomembranes and Nutrition. Vol. 195,p319-332. Colleque INSERM.

167.Lopez S, Burlet H, and Desbuquois B. Mechanisms of up-regulation of the liver insulin receptor in chronically hypoinsulinemic rats: assessment of receptor endocytosis. Mol Cell Endocrinol, 1991;82:159-164.

168.Fickova, M., Hubert, P., Klimes, I., Staedel, C., Cremel, G., Bohov, P., and Macho, L. Dietary fish oil and olive oil improve the liver insulin receptor tyrosine kinase activity in high sucrose fed rats. (1994) Endocrinol. Regul.,28(4):187-197.

169.McCallum, C.D., and Epand, R.M. Insulin receptor autophosphorylation and signaling is altered by modulation of membrane physical properties. (1995) Biochem.,34(6):1815-1824.

170.Blake, W.L., and Clarke, S.D. Suppression of rat hepatic fatty acid synthase and S14 gene transcription by polyunsaturated fatty acids. (1990) J. Nutr.,120:1727-1729.

171.Katsurada, A., Iritani, N., Fukuda, H., Matsumura, Y., Nishimoto, N., Noguchi, T., and Tanaka, T. Effects of nutrients and hormones on transcriptional and post-transcriptional regulation of fatty acid synthase in the liver. (1990) Eur. J. Biochem.,190:435-441.

172.Ntambi, J.M. The regulation of stearoyl-CoA desaturase (SCD). (1995) Prog. Lipid Res.,34(2):139-150.

173.Clarke, S.D., and Jump, D.B. Regulation of gene transcription by polyunsaturated fatty acids. (1993) Prog. Lipid Res.,32(2):139-149.

174.Iritani, N., Hosomi, H., Fukuda, H., and Ikeda, H. Polyunsaturated fatty acid regulation of lipogenic enzyme gene expression in liver of genetically obese rats. (1995) Biochim. Biophys. Acta,1255:1-8.

175.Dugail, I. Quignard-Boulange, A., Le Liepvre, X., and Ardouin, B. Gene expression of lipid storage-related enzymes in adipose tissue of the genetically obese Zucker rat: coordinate increase in transcriptional activity and potentiation by hyperinsulinemia. (1992) Biochem. J.,282(3):607-611.

176.Maury, J., Issad, T., Perdereau, D., Gouhot, B., Ferre, P., and Girard, J. Effect of acarbose on glucose homeostasis, lipogenesis, and lipogenic enzyme gene expression in adipose tissue of weaned rats. (1993) Diabetol.,36(6):503-509.

177.Cheema, S.K., and Clandinin, M.T. Diet fat alters the expression of genes for enzymes of lipogenesis in lean and obese mice. (1996) Biochim. Biophys. Acta,1299:284-288.

178.Doiron, B., Cuif, M.-H., Kahn, A., and Diaz-Guerra, M.-J. (1994) J. Biol. Chem.,269:10213-10216.

179.Foufelle, F., Gouhot, B., Begorier, J.-P., Perdereau, D., Girard, J., and Ferre, P. (1992) J. Biol. Chem.,267:20543-20546.

180.Mariash, C.N., and Oppenheimer, J.H. (1983) Metabolism,33:545-552.

181.Towle, H.C. Metabolic regulation of gene transcription in mammals. (1995) 270(40):23235-23238.

# CHAPTER II

# INSULIN RECEPTOR ACCUMULATION IN THE MOUSE HEPATOCYTE CELL NUCLEUS FOLLOWING *IN VIVO* STIMULATION WITH ORAL GLUCOSE

## *INTRODUCTION*

The insulin receptor spans the plasma membrane of insulin-responsive cells. The α-subunit contains an insulin-binding domain and is located on the outside face of the membrane (1, 2). The β-subunit has both extracellular and cytoplasmic domains, and a membrane-spanning domain (1, 2). It also contains a kinase that is capable of catalyzing tyrosine phosphorylation both within the receptor itself and towards cellular substrates (3). The insulin receptor tyrosine kinase is activated by ligand binding which triggers autophosphorylation on specific tyrosine residues in a ATP-binding region called the regulatory domain. The phosphorylation of these residues is important for stimulating kinase activity towards intracellular substrates (3). Upon autophosphorylation, the hormone receptor complex migrates to non-villous domains on the cell surface (4). Within these regions the receptor is able to anchor to clathrin-coated pits which invaginate and become internalized in a process called endocytosis (5). The insulin receptor, like other internalized receptors including the low-density lipoprotein receptor, contains at least one tetrapeptide motif which allows it to associate with clathrin-coated pits (6). In the insulin receptor this motif, made up of asparagine, proline, glutamate, and tyrosine (NPEY), is found proximal to the intracellular portion of the membrane-spanning domain and is referred to as the juxtamembrane domain. Kinase activation is not required for anchoring to clathrin-coated pits, instead the NPEY domain plays a necessary role (7). The insulin receptor-ligand complex is delivered by endocytosis to vesicles and tubules that make up the endosomal apparatus. The acidification of endosomes causes the receptor-ligand complex to dissociate. Insulin molecules are targeted to lysosomes for degradation, receptor molecules are recycled back to the cell surface where they are reused (5). Little is known about the mechanisms involved in the ligand-receptor dissociation and sorting in endosomes except that this process requires calcium (8). Recent information suggests that receptor dephosphorylation in endosomes is necessary for ligand-receptor dissociation, when this is impaired receptors are targeted to lysosomes and recycling is slow and inefficient (9).

Internalization of the insulin receptor functions to clear and inactivate insulin, thus attenuating the signal, and to modulate the number of surface insulin receptors, and therefore the sensitivity of the cell to insulin, a process is referred to as downregulation (10). Another function of internalization may be the transmission of the insulin signal by allowing the activated receptor to gain access to plasma-membrane inaccessible substrates. Of interest is the finding that the insulin receptor tyrosine kinase within the endosomal apparatus is four- to six-fold more active than that within the plasma membrane (11,12).

Several attempts have been made to determine the subcellular compartments through which the insulin receptor moves during recycling. Using photoactive insulin analogs that covalently attach to the insulin receptor, insulin-receptor complexes have been followed by electron microscopic autoradiography. These complexes have been found moving from the plasma membrane into endosomes, though lysosomes, and back though endosomes before moving back to the plasma membrane (13). It is important to determine if the path taken by the receptor-ligand complexes is that followed under normal conditions or that taken when ligand dissociation is prevented.

In some studies tracking the insulin-receptor complex within the cell, insulin receptors have been localized on the nucleus of hepatocytes (14-18) and adipocytes (19,20). In these studies insulin binding sites were indicated by the presence of specific [$^{125}$I]-insulin binding to isolated nuclei. In the model proposed above, the steps of insulin receptor ligand-induced intracellular cycling can be outlined simply as: 1) plasma membrane-ligand free, 2) plasma-membrane-ligand bound, 3) endosomal apparatus-ligand bound, 4) endosomal apparatus-ligand free, 5) intracellular compartment-ligand free and finally 6) plasma membrane-ligand free. Using this model, insulin receptors were expected to be associated with nuclei *following* ligand stimulation. In most of these studies (15,16,18), insulin receptors was detected in cell nuclei of animals in the fed state. However in one study (17), insulin receptors were found in the cell nuclei of animals in the fasted state, suggesting that the insulin receptor may also reside in the nuclei under basal conditions. Time courses of insulin receptor accumulation in the cell nucleus has been reported *in vitro*, using adipocytes (19,20) and hepatocytes (14). All of these studies report no detection of receptor under non-stimulated conditions, or under situations where ligand-receptor recycling was impaired. These findings support the hypothesis that insulin receptors are found in the cell nucleus in a period *following* ligand stimulation.

The objective of this study was to determine whether insulin receptor ligand-induced recycling and association with the cell nucleus can be observed *in vivo*. The experimental design involved inducing insulin stimulation via an oral glucose gavage given to mice in the fasted state. Measurement of insulin binding to nuclei was determined in fasted mice and in nuclei isolated from mice up to 180 minutes following treatment by oral glucose gavage.

## *MATERIALS AND METHODS*

### ***Animal Care and Treatment***

Eight week old C57 Black 6 mice (Jackson Laboratories, Bar Harbour, Maine, USA), were fed *ad libitum*, Richmond Laboratory Standard Rodent Diet 5001® (PMI Feeds, Inc., St. Louis, MO, USA). Animals were housed in a temperature and humidity controlled room with a 12 hour light/dark cycle. Animals were housed in groups of four to six in polypropylene cages. The protocol was approved by the University of Alberta Animal Welfare Committee.

Prior to an experimental day, animals were fasted for 12 h beginning at 8:00 p.m. and ending at 8:00 am, on the day of the experiment. Mice were then given a standard dosage of glucose of 3 g/kg body weight oral gavage (21). At selected times (0, 15, 30, 60, 90, 120, and 180 min) following gavage, mice were anesthetized with Halothane, (Halocarbon Laboratories, Rivers Edge, NJ, U.S.A.), and blood was collected by cardiac puncture. Animals were sacrificed by cervical dislocation and the liver was immediately removed. Five mice were used for analysis for each of the seven selected lengths of time following oral glucose gavage.

### *Preparation of Liver Nuclei*

Liver nuclei were isolated according to the procedure by Blobel and Potter (22) with the following modifications. Immediately after removal each liver was minced in ice cold homogenization buffer containing 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl flouride (PMSF), 1 mM benzamidine-HCl, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 0.0044 trypsin inhibitor units (TIU)/ml aprotinin. Liver was homogenized using a Potter-Elvehjem homogenizer (A.H. Thomas, Philadelphia, Pennsylvania), using 2 up-down stokes, and then filtered through 5 layers of cheesecloth. A crude nuclear pellet was obtained by centrifugation for 20 min at 1 450 x $g_{max}$, using a JA 20 rotor (Beckman Instruments, Palo Alto, CA, U.S.A). The supernatant was used for plasma membrane purification. The pellet was suspended in homogenization buffer (2 ml/g liver). This buffer containing 2.4 M sucrose was added to the suspension until the sucrose concentration was 1.6 M. The suspension was then centrifuged at 70 900 x $g_{av}$ for 70 min using a SW28 rotor (Beckman). The pellet containing nuclei was suspended in nuclei isolation buffer containing 50 mM Tris-HCl, 250 mM sucrose, 5 mM $MgCl_2$, 2.5 KCl , 1 mM PMSF, 1 mM benzamidine-HCl, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 0.0044 TIU/ml aprotinin. One volume of nuclei isolation buffer containing 2.2 M sucrose was added and the resulting suspension was overlayed over 5 ml of this 2.2 M sucrose containing buffer. Nuclei pellets were pelleted by centrifugation at 70 900 x $g_{av}$ for 60 min. Pellets were suspended in nuclei isolation buffer and one volume of 2.2 M sucrose containing nuclei isolation buffer was added. Finally the nuclei suspension was overlayed on 5 ml of 2.2 M sucrose containing nuclei isolation buffer and centrifugation was repeated. The pellet containing purified nuclei, was resuspended in 1 ml/g of nuclei isolation buffer.

### *Insulin binding to isolated nuclei*

Insulin binding assays (23) were completed using freshly isolated nuclei preparations. Insulin binding activity of nuclei was measured by incubating preparations containing 200 μg protein for 2 hours at 25$^0$C with 4 nM of $^{125}$I-labeled porcine insulin (40 μCi/ug) (NEN Life Science Products, Boston, MA, U.S.A.) in a final volume of 200 μl of buffer containing 62.5 mM sucrose, 5 mM Tris-HCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, pH 7.5, in the presence (20 μg/ml) and absence of excess unlabelled porcine insulin. The receptor-$^{125}$I-labeled insulin complex was separated from free $^{125}$I-insulin by adding 0.6 ml 50 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 0.1 % w/v rabbit γ-globulin, 10 % w/v polyethylene glycol 8000, and incubating for 15 min on ice before centrifugation at 14 000 x $g_{max}$ for 5 min. The supernatant containing unbound insulin, was aspirated and the pellets counted using a Gamma 8000 (Beckman Instruments) counter. The radioactivity precipitated in the presence of unlabelled insulin was considered non-specific binding. Specific binding was calculated by subtracting non-specific binding from the radioactivity precipitated in the absence of unlabelled insulin, and was expressed as fmol of insulin bound per mg protein.

### *Glucose and Insulin Determination*

Immediately after blood collection, serum was prepared by centrifugation at 2000 x $g_{max}$ for 10 min using a JS-4.3 rotor (Beckman). Serum was frozen at -70$^0$C until analysis. Serum glucose was determined using Glucose Trinder Reagent (Sigma Diagnostics, St. Louis, MO, U.S.A.) for the enzymatic determination of glucose. Insulin was measured using a commercial double antibody immunoassay for rat insulin (Linko Research, St. Louis, MO, USA).

### *Purification of Mouse Plasma Membrane*

Liver plasma membranes were prepared from fasted mice using a modified procedure (24). The post-nuclei supernatant was centrifuged at 8700 $g_{max}$ to pellet mitochondria. The supernatant was centrifuged at 105 000 $g_{max}$ for 30 min using a Ti45 rotor (Beckman Instruments) to pellet plasma membranes. This pellet was suspended in plasma membrane isolation buffer, 5 mM Tris-HCl pH 7.5, 0.25 M sucrose, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM PMSF, 1 mM benzamidine-HCl, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 0.0044 TIU/ml aprotinin. This buffer, (1.04 volumes), containing 2.4 M sucrose was added so that the final sucrose concentration was 1.35 M. This suspension was aliquoted into centrifuge tubes and overlayed with 0.3 volume of buffer A. Tubes were centrifuged at 231 000 x $g_{av}$ for 30 min using a Ti70.1 rotor (Beckman), and plasma membranes collected at the interface. Plasma membranes were used as a positive control in insulin binding assays and for determining the membrane contamination of nuclei preparations.

### *Protein Content and 5'Nucleotidase Activity in Isolated Nuclei*

Protein content of isolated nuclei and plasma membrane was determined (25). Samples from freshly isolated nuclei and plasma membrane were used to assay membrane 5'nucleotidase activity (26). Contamination of nuclei preparations by plasma membrane was assessed by calculating the relative specific activity of 5'nucleotidase found in isolated nuclei versus that of plasma membrane (24). Specific activity of 5'nucleotidase was the amount of nmol of phosphate measured after 20 min using 50 μg protein. Relative specific activity was calculated by dividing the specific activity of nuclei by the specific activity of plasma membrane and multiplying by 100.

### *Statistical Analysis*

The difference between the time treatments following oral glucose gavage were assessed using analysis of variance procedures (SAS Version 6.11, SAS Inc., Cary, NC, USA) (27) A significant difference between treatments was determined by a Duncan's multiple range test ($p<0.05$) (28). The correlation between serum glucose, serum insulin, and insulin binding to isolated nuclei at basal conditions and following oral glucose gavage was tested by calculating Pearson correlation coefficients using SAS.

## *RESULTS*

### *Animal Characteristics*

Eight week male mice used in this experiment had average body weights of 28.4 ± 0.3 g and liver weights of 1.29 ± 0.02 g. There were no differences in these measures between mice treated for different time periods following oral glucose gavage.

### *Isolation of Nuclei*

The amount of contamination of isolated nuclei by plasma membrane was assessed by marker enzyme analysis using 5'nucleotidase, a plasma membrane marker. The relative specific activity of nuclear membrane 5'nucleotidase compared to the activity found in plasma membrane averaged 3.8 ± 1.2 % (**Table 2.1**). In preliminary experiments there was no difference found between percent contamination of nuclei by plasma membrane isolated from hepatocytes from mice fed *ad libitum* (5.5 ± 2.3 %) and mice fasted for 12 hours (3.52 ± 1.3%). Inspection by bright field microscopy (**Figure 2.1**), revealed intact nuclei with no contamination by cellular debris.

### *Serum Glucose and Insulin Measurements*

Following the 12 h fast, mice exhibited a serum glucose concentration of 8.63 ± 1.47 mM. The effect of oral glucose gavage on glucose levels indicated that, within the time periods tested, serum glucose levels peaked at 24.5 ± 1.3 mM within 15 min; after that time glucose levels decreased and reached basal values by 180 min following oral glucose (**Figure 2.2**). Serum insulin levels in fasted mice were 19 ± 4.6 pM. The effect of oral glucose on serum insulin levels is illustrated (**Figure 2.3**). A significant increase from basal serum insulin levels occurred within 15 min following oral glucose, and levels peaked at 30 min following gavage. The rise in serum glucose and insulin suggested that insulin stimulation was achieved *in vivo* within 15 min following oral glucose.

### *Insulin Binding*

The effect of oral glucose administration on insulin binding to isolated nuclei is illustrated (**Figure 2.4**). Insulin binding to nuclei isolated at each time following oral glucose is expressed as a percentage change from basal (0 min) conditions. There was a 206 ± 7 % increase from basal at 15 min ($p<0.05$), a 219 ± 84% increase from basal at 30 min ($p<0.05$), and a 259 ± 211% increase from basal at 60 min (non significant), following oral glucose. At 90 to 180 min following oral glucose gavage insulin binding to nuclei decreased to that of basal levels.

### *Relationship between serum glucose and insulin and insulin binding to isolated nuclei*

As expected a significant positive correlation was observed between the serum glucose and insulin concentrations following oral glucose gavage ($r^2 = 0.71$ $P < 0.0001$). Insulin binding to isolated hepatocyte nuclei following oral glucose gavage was also significantly correlated with serum glucose and with serum insulin concentrations ($r^2 = 0.55$ $P < 0.001$) and ($r^2 = 0.44$ $P < 0.01$) respectively.

## *DISCUSSION*

Oral glucose treatment of 3 g/kg body weight rapidly increases serum glucose and insulin levels in fasted mice. This increase is coincident with an increase in specific insulin binding to the hepatocyte nucleus. A significant relationship was observed between serum glucose and insulin concentrations and insulin binding to hepatocyte nuclei following oral glucose gavage. This relationship suggests that *in vivo* stimulation of hepatocytes with insulin causes a time-dependant increase in insulin receptor internalization and translocation to the cell nucleus.

Increase in plasma glucose and insulin was rapid and transient, plasma insulin levels peaked at 30 min following oral glucose. Mice appeared to have poor glucose tolerance following glucose challenge. Fasting has been demonstrated to impair glucose tolerance (29). Glucose tolerance in mice of this age has been reported to be impaired which might reflect a metabolic bias to prevent hypoglycemia in early life (29,30). In addition, the transition at weaning (3 weeks of age) from a high fat low carbohydrate diet to a solid diet rich in carbohydrate may create a temporary imbalance in plasma glucose regulation (31).

Insulin binding to the cell nucleus peaked at levels two fold higher than basal levels at 30 min following oral glucose. That insulin binding to the hepatocyte nucleus is coincident with the changes in serum insulin suggests that the kinetics of internalization and translocation to the nucleus is triggered by ligand stimulation, that the translocation is rapid, and that levels of receptor are reduced in the nucleus in the absence of continuous stimulation. The finding that insulin receptor translocation to the nucleus occurred upon ligand stimulation, is consistent with several demonstrations that insulin receptor internalization is largely ligand-induced (7, 12, 32-34). Insulin receptor internalization in intracellular compartments has been reported to have a $t_{1/2}$ of 2 to 10 min in insulin-stimulated cultured cells (32-34). The kinetics of insulin accumulation in the nucleus has been reported in only two publications, both using cells in culture. In hepatocytes (14), maximum levels of insulin receptor binding to the nucleus was observed 1 hr following insulin stimulation. In adipocytes (20), maximum levels of insulin receptor detected by immunoblotting were observed 5 min following stimulation. The results in the present study indicate a shorter $t_{1/2}$ than that reported in hepatocytes. As the present study was done *in vivo*, the rapid internalization of the insulin receptor can be taken to approximate the physiological condition. The differences in internalization rates observed in hepatocytes may also reflect subtle differences in technique used to measure the insulin receptor. Both studies used [$^{125}$I]-labeled insulin as a probe, however, in the earlier study (14), [$^{125}$I]-labeled insulin was covalently attached to receptor by photoaffinity labeling. It is possible that the receptor-ligand complex may not have been processed in a manner comparable to that of the non-covalently occupied insulin receptor.

Several lines of evidence suggest that the finding of nuclear insulin receptors is not due to contamination by plasma membrane during cell fractionation. 5`nucleotidase marker enzyme analysis suggests that the purity of isolated nuclei in the present study is 96 to 100 %. Nuclear preparations from *ad libitum* fed animals had similar plasma membrane contamination (5.5 ± 2.3 %) when compared to those obtained from fasted animals (3.5 ± 1.3 %). Thus feeding was not found to influence purification of nuclei, which suggests that increased nuclear insulin binding associated with glucose-treated animals could not be due to increases in plasma contamination. Microscopy indicated that the nuclei isolated were both intact and free of cellular debris.

Insulin-like growth factor (IGF) and insulin are known to produce similar biological responses. Thus it is possible that insulin stimulation produced a cross activation of the IGF receptor and these receptors were detected in the isolated nucleus. However IGF receptors have a low affinity to insulin and only bind insulin at high concentrations (35). The peak serum insulin concentration achieved by oral glucose administration was 500 pM which is far below the concentration required for IGF receptor activation in the plasma membrane (35). This rules out the possibility that insulin induced nuclear translocation of the IGF receptor.

In conclusion, an increase in nuclear insulin binding was induced by a rise in serum insulin following oral glucose gavage. The translocation of insulin receptors to the nucleus appeared to be both time dependent and ligand dependent. Demonstration of nuclear accumulation of insulin receptor following ligand stimulation *in vivo* may have physiological implications for mechanisms involved in insulin signaling to the cell nucleus.

## *REFERENCES*

1. Zick, Y. The insulin receptor: Structure and function. (1989) Crit. Rev. Biochem. Mol. Biol.,24(3):217-269.

2. Denton, R.M. Early events in insulin action. In: Advances in Cyclic Nucleotide and Protein Phosphorylation Research, vol 20, Greengard, P., and Robison, G.A., Eds., Raven Press, New York, 1986, 293-341.

3. Kasuga, M., Karlsson, F.A., and Kahn, C.R. Insulin stimulates the phosphorylation of the 95, 000 dalton subunit of its own receptor. Science,215:185-187.

4. Carpentier, J.L., Paccaud, J.P., Gorden, P., Rutter, W.J., and Orci, L. Insulin-induced surface redistribution regulates internalization of the insulin receptor and requires its autophosphorylation. (1992) Proc. Natl. Acad. Sci. U.S.A.,89:162-166.

5. Carpentier, J.L. The journey of the insulin receptor into the cell: from cellular biology

to pathophysiology. Histochem., 100:169-184.

6. Chen, W.J., Goldstein, J.L., and Brown, M.S. (1990) J. Biol. Chem., 265:3116:3123.

7. Backer, J.M., Shoelson, S.E., Haring, E., and White, M.F. Insulin receptors internalize by a rapid, saturable pathway requiring receptor autophosphorylation and a juxtamembrane region. (1992) J.Cell. Biol.,118:831-839.

8. Carpentier, J.L., Gordon, P., and Lew, D.P. Calcium ions are required for the intracellular routing of insulin and its receptor. Exp. Cell. Res.,198:144-149.

9. Carpentier, J.L. Insulin receptor internalization: molecular mechanisms and physiopathological implications. (1994) Diabetiologia,37(suppl2):s117-s124.

10. Knutson, V.P., Ronnet, G.V., and Lane, M.O. Rapid reversible internalization of rapid surface receptor "correlation with insulin-induced downregulation". (1983) J. Biol. Chem.,258:12139-12142.

11. Burgess, J.W., Bevan, A.P., and Bergeron, J.J.M. Pharmacological doses of insulin equalize insulin receptor phosphotyrosine contest but not tyrosine kinase activity in plasmalemmal and endosomal membranes. (1992) Biochem. Cell. Biol.,70:1151-1158.

12. Kublaoui, B., Lee, J., and Pilch, P.F. Dynamics of signalling during insulin-stimulated endocytosis of its receptor in adipocytes. (1995) J. Biol. Chem.,270(1):59-65.

13. Carpentier, J.L., Fehlmann, M., VanObberghen, E., Gorden, P., and Orci, L. Insulin receptor internalization and recycling: mechanism and significance. (1985) Biochimie,67:1143-1145.

14. Podlecki, D.A., Smith, R.M., Kao, M., Tsai, P., Huecksteadt, T., Brandenburg, D., Sasher, R.S., Jarett, L., and Olefsky, J.M. Nuclear translocation of the insulin receptor. A possible mediator of insulin's long term effects. (1987) J. Biol. Chem., 292:3362-3363.

15. Wong, K.Y., Hawley, D., Vigneri, R., Goldfine, I.D. Comparison of solubilization and purified plasma membrane and nuclear insulin receptors. (1988) Biochemistry, 27:375-379.

16. Vigneri, R., Goldfine, I.D., Wong, K.Y., Smith, G.J., and Pezzino, V. The Nuclear Envelope. The major site of insulin binding in rat liver nuclei. (1978) J. Biol. Chem., 253(7);2098-2103.

17. Horvat, A. Insulin binding sites on rat liver nuclear membranes: Biochemical and

immunoflourescent studies. (1978) J. Cell. Physiol., 97:37-48.

18. Cheema, S.K., Venkatraman, J.T., and Clandinin, M.T. Insulin binding to liver nuclei from lean and obese mice is altered by dietary fat. (1992) B.B.A., 1117:37-41.

19. Smith, R.M., and Jarett, L. Ultrastructural evidence for the accumulation of insulin in nuclei of intact 3t3-L1 adipocytes by an insulin-receptor mediated process. (1987) Proc., Natl., Acad., Sci., U.S.A., 84:459-463.

20. Kim, S.-J., and Kahn, C.R. Insulin induces rapid accumulation of insulin receptors and increases tyrosine kinase activity in the nucleus of cultures adipocytes. (1993) J. Cell. Physiol., 157:217-228.

21. Flatt, P.R., and Bailey, C.J. Abnormal plasma glucose and insulin responses in heterozygous lean (*ob*/+) mice. (1981) Diabetol.,20:573-577.

22. Blobel, G., and Potter, V.R. Nuclei from rat liver: Isolation method that combines purity with high yield. (1966) Science, 154:1662-1665.

23. Fujita-Yamaguchi, Y., Choi, S., Sakamato, Y., and Itakura, K. Purification of insulin receptor with full binding activity. (1983) J. Biol. Chem., 258(8):5045-5049.

24. Fleischer, S., and Kervina, M. Subcellular fractionation of rat liver. (1974) Meth. Enzymol.,31:6-41.

25. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the folin phenol reagent. (1951) J. Biol. Chem.,193:265-275.

26. Aronson, N.N.Jr., and Touster, O. Isolation of rat liver plasma membrane fragments in isotonic sucrose. (1974) Meth. Enzymol.,31:90-102.

27. Steel, R.G.D., and Torrie, J.H. Principles and procedures of statistics, a biometrical approach 2nd edition. C. Napier, and J.W. Maisel eds. (1980) McGraw-Hill, Inc., New York, USA.

28. Duncan, D.B. Multiple range and multiple *F* tests. (1955) Biom.,11:1-42.

29. Flatt, P.R., and Bailey, C.J. Development of glucose intolerance and impaired plasma insulin response to glucose in obese hyperglycemic (ob/ob) mice. (1981) Horm. Metab. Res.,13:556-560.

30. Beloff-Chain, A., Freund, N., and Rookledge, K.A. Blood glucose and serum insulin levels in lean and genetically obese mice. (1975) Horm. Metab. Res.,7:374-378.

31. Dubuc, P.U. The development of obesity, hyperinsulinemia, and hyperglycemia in

ob/ob mice. (1976) Metab.,25:1567-1574.

32. Backer, J.M., Kahn, C.R., and White, M.F. Tyrosine phosphorylation of the insulin receptor during insulin-stimulated internalization in rat hepatoma cells. (1989) J. Biol. Chem.,264:1694-1701.

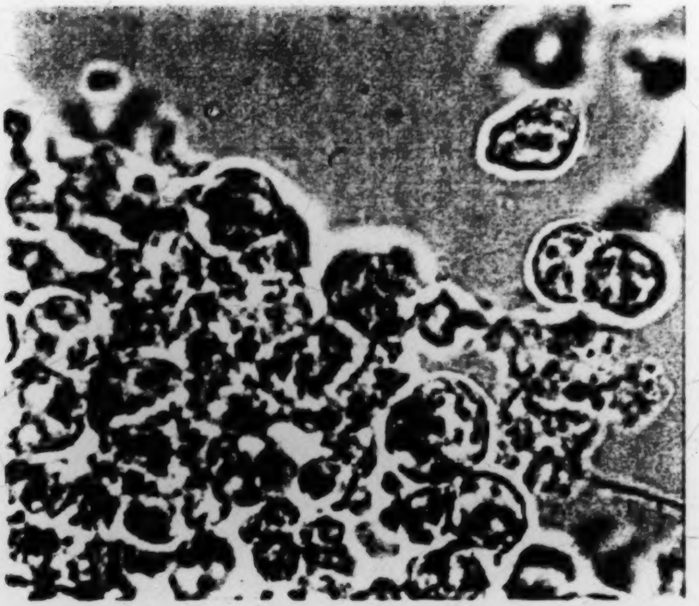
33. Cousin, J.L., Samson, M., Pilch, P.F., and Fehlman, M. Internalization of insulin receptors and HLA antigens in human hepatoma cells. (1987) Biochem. J.,242:403-410.

34. Marshall, S. Kinetics of insulin receptor internalization and recycling in adipocytes. Shunting of receptors to a degradative pathway by inhibitors of recycling. (1985) J. Biol. Chem.,260(7):4136-4144.

35. Megyesi, K., Kahn, C.R., Roth, J., Froesch, E. R., Humbel, R.E., et al. Insulin and nonsuppressible insulin-like activity (NSILA-s): evidence for separate plasma membrane receptor sites. (1974) Biochem. Biophys. Res. Commun.,57:307-315.

36. Rechler, M.M., and Nissley, S.P. Receptors for insulin-like growth factors. In Receptors for Polypeptide Hormones, ed. B.I. Posner, New York, Dekker.

**Figure 2.1 Isolated nuclei.** A representative of nuclei isolated from mice hepatocytes. Nuclei were visualized by bright field microscopy at 1 000 x magnification using a Dialux 20 EB microscope, Leitz, Berlin, Germany.



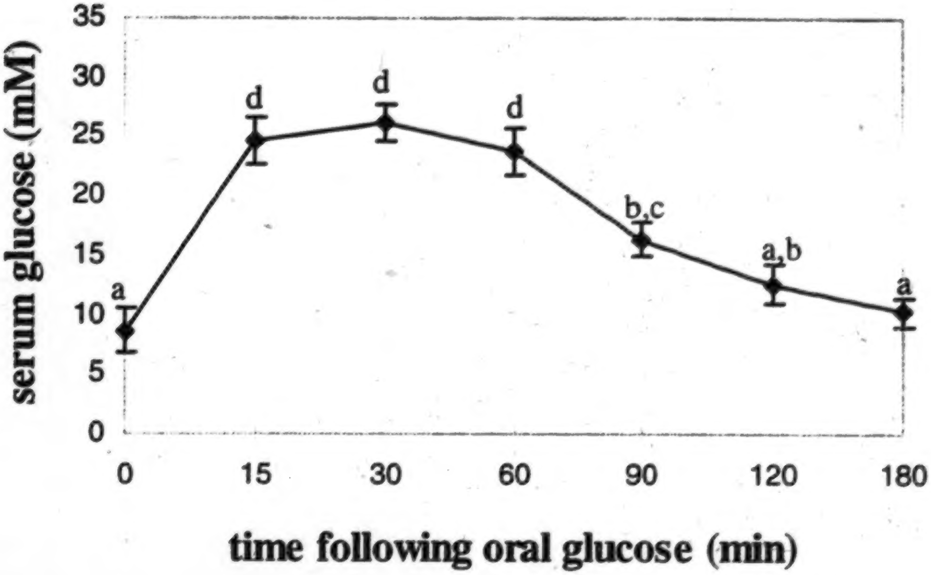
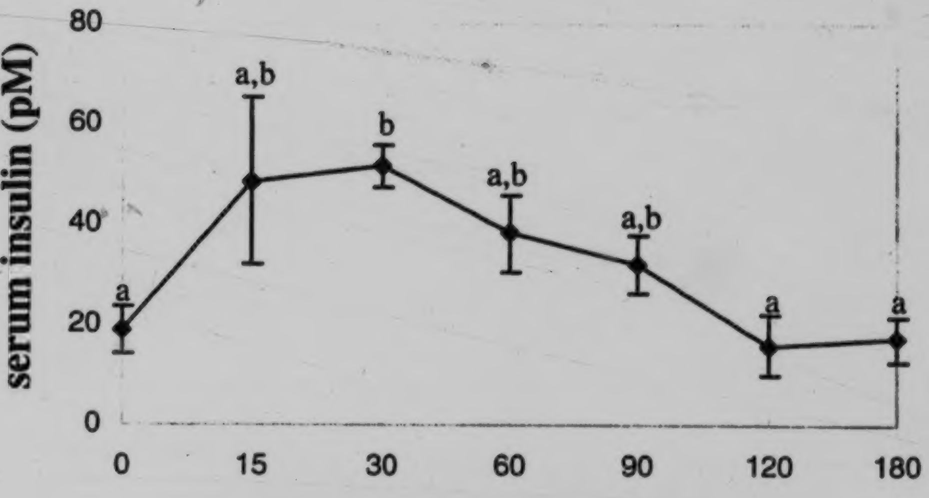


**Figure 2.2 Serum glucose response to oral glucose gavage in eight week old male mice.** Mice were fasted for 12 h before oral gavage with 3 g/kg body glucose. After selected time periods following treatment, mice were anesthetized and blood removed by cardiac puncture. Serum was prepared and assayed for glucose, which was measured in mM. Data represents mean ± standard error of the mean (n = 6 for each timepoint). Values labeled with different letters are significantly different ($p < 0.05$).



**time following oral glucose (min)**

**Figure 2.3 Serum insulin response to oral glucose gavage in eight week old male mice.** Mice were fasted for 12 h before treatment with 3 g/kg body glucose. After selected time periods following treatment, mice were anesthetized and blood removed by cardiac puncture. Serum was prepared and assayed for insulin which was measured in pM. Data represents mean ± standard error of the mean (n = 6 for each timepoint). Values labeled with different letters are significantly different ($p < 0.05$).

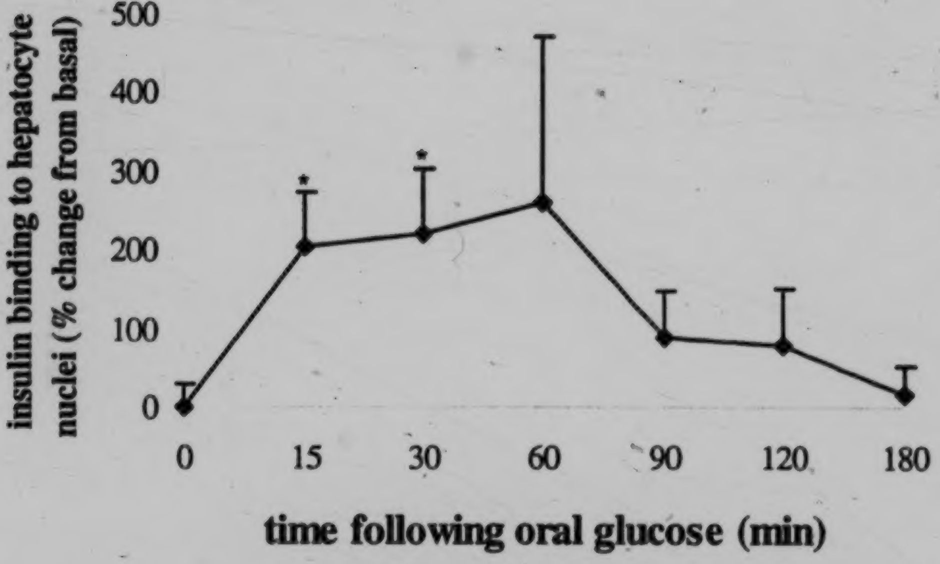


**Figure 2.4 The response of [$^{125}$I]-insulin binding to isolated hepatocyte nuclei following oral glucose gavage.** Mice were fasted for 12 hours before treatment with 3 g/kg body weight glucose. After selected time periods following treatment, liver was removed and nuclei isolated. 200 ug of nuclei extract was used in an insulin binding assay. Specific insulin binding was measured in fmol insulin bound per mg protein and expressed as a percentage change from basal conditions (0 min= 159.7 ± 65.2). Data represents mean ± standard error (n = 6 for each timepoint). Comparisons were made between selected time following glucose gavage and basal condition. Values with (*) are significantly different from basal ($p < 0.05$).

TABLE 1 Isolation of Nuclei and Plasma Membrane from Mouse Liver

| | 5'Nucleotidase | | | DNA | |
|---|---|---|---|---|---|
| | Total Activity (nmol/min) | Specific Activity (nmol/min/mg protein) | Fold Purification | Total DNA (mg per 3 livers) | Yield of DNA |
| Homogenate | 40769 ± 2993 | 30.1 ±1.3 | 1 | 50.6 ± 3.1 | 100 |
| Plasma Membrane | 4292 ± 1008 | 167.8 ± 36.2 | 5.5 ± 1.0 | N.D. | |
| Nuclei | 912 ± 212 | 5.7 ± 1.2 | 0.2 ± 0.0 | 4.2 ± 0.4 | 8.3 ± 0.5 |

Values are mean ± standard error of the mean (n=8). Four mice fed ad libitum and four mice fasted for 12 hours were used for plasma membrane and nuclei purification and marker enzyme analysis. Statistical analysis was done using Student's *t*-test ($p < 0.05$). Values were not found to be different between treatment and therefore were pooled. Specific activity was calculated as the ratio of total enzyme activity to total protein. Fold purification was calculated as the specific activity of the fraction of interest divided by the specific activity of the homogenate. N.D = not determined.

# CHAPTER III

# OBESE MICE HAVE HIGHER INSULIN RECEPTOR LEVELS IN THE HEPATOCYTE CELL NUCLEUS FOLLOWING INSULIN STIMULATION *IN VIVO* WITH AN ORAL GLUCOSE MEAL.

## *INTRODUCTION*

Obesity and non-insulin dependent diabetes mellitus (NIDDM), share characteristic features including insulin resistance and fasting hyperglycemia, which suggests that obesity is strongly associated with insulin resistance (1). NIDDM is more likely to develop as the degree and the duration of obesity increases and if the obesity is centrally located (1). However, whether the relationship between obesity and insulin resistance is causative is not known (2).

At the cellular level, obesity has been suggested to cause abnormalities in major phases of the insulin signaling pathway: ligand-receptor binding and activation, post-receptor signaling, and hormone-effector systems (3). In humans, obesity is associated with a reduction in insulin receptor number found in muscle and adipose tissue (4,5). In rodent models of obesity, insulin receptor levels are reduced in liver (6-9). The decrease in insulin receptors in obesity may be caused by a reduction in cell-density, or number of insulin receptors per surface area of the cell, that occurs as the size of the cell increases (10). It has been suggested that the increase in circulating free fatty acids that accompany obesity impair insulin binding to its receptor (9,11). The decrease in insulin receptor in obesity may be induced by hyperinsulinemia that occurs with the disease. High plasma insulin concentrations are known to regulate cell surface receptor numbers, a phenomenon termed down-regulation. Thus as the amount of ligand binding to the insulin receptor increases the rate of ligand-induced internalization is increased resulting in lower receptor levels on the cell surface (12-14).

Obesity has an effect on the activity of the insulin receptor tyrosine kinase. In humans (4,15,16) and in animal models (17-19), obesity is associated with a defect in β-subunit autophosphorylation and in the ability of receptor to phosphorylate exogenous substrates. Studies have demonstrated that post-translational modification in the insulin receptor β-subunit, including dephosphorylation of tyrosine residues (20) and phosphorylation of serine residues, is increased in obesity and may be responsible for the defect in kinase activity that occurs with the disease (21,22).

Studies have demonstrated that the insulin receptor becomes associated with intracellular membranes following ligand stimulation and that it catalyzes phosphorylation of substrates such as IRS-1 from this location (23,24). In obese compared to lean subjects, defects in insulin receptor internalization (8,25,26) and processing have been demonstrated (27) by measuring the intracellular pool of receptor following ligand stimulation. Increasing concentrations of free fatty acids impair internalization of insulin in a dose-dependent manner in cultured hepatocytes (11). In this study (11), the defect appeared to be related to a reduced ability to process receptor-bound insulin and not related to the numbers of receptors available.

Recent studies suggest that insulin signaling to the nucleus may involve internalization and translocation of the insulin receptor to the nucleus upon ligand stimulation (28-31). In obese compared to lean mice, insulin binding to hepatocyte nuclear membranes is reduced (32). The objective of this study is to determine if the accumulation of insulin receptors in the hepatocyte nuclei upon ligand stimulation is impaired in an animal model of obesity. The experimental design included stimulation *in vivo* by administering an oral glucose gavage to fasted lean and obese mice. Hepatocyte nuclei were isolated at selected times following oral glucose gavage and levels of insulin receptor in the nuclei were determined by $^{125}$I-insulin binding and by Western blotting assays.

## *MATERIALS AND METHODS*

### ***Animal Care and Treatment -Lean and Obese Mice***

Eight week old C57 Black 6 J lean (C57BL/6J) (+/+) and obese (ob/ob) mice (Jackson Laboratories, Bar Harbour, Maine, U.S.A), were housed in groups of four to six in polypropylene cages. Mice were given free access to a rodent diet (Laboratory Standard Rodent Diet 5001, PMI Feeds, Inc., St. Louis, MO, USA). Prior to an experimental day, animals were fasted for 12 h beginning at 8:00 p.m. and ending at 8:00 am, on the day of the experiment. Mice were then given a 3 g/kg body weight dose of glucose by oral gavage. At selected times (0, 15, 30, 60, 90, 120, and 180 min) following gavage, mice were anesthetized with Halothane, (Halocarbon Laboratories, Rivers Edge, NJ, U.S.A.), and blood was collected by cardiac puncture. Animals were sacrificed by cervical dislocation and liver was immediately removed. Five mice were used for analysis for each of the seven selected periods of time following oral glucose gavage.

***Preparation of liver nuclei***

Liver nuclei were isolated according to the procedure by Blobel and Potter (33) with the following modifications. Immediately after removal each liver was minced in ice cold homogenization buffer containing 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM PMSF, 1 mM benzamidine-HCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.0044 TIU/ml aprotinin. Liver was homogenized using a Potter-Elvehjem homogenizer, using 2 up-down stokes, and then filtered through 5 layers of cheesecloth. A crude nuclear pellet was obtained by centrifugation for 20 min at 1 450 $g_{max}$, using a JA 20 rotor (Beckman Instruments, Palo Alto, CA, USA). The supernatant was used for plasma membrane purification. The pellet was suspended in homogenization buffer (2 ml/g liver). This buffer containing 2.4 M sucrose was added to the suspension until the sucrose concentration was 1.6 M. The suspension was then centrifuged at 70 900 $g_{av}$ for 70 min using a SW28 rotor (Beckman). The pellet containing nuclei was suspended in nuclei isolation buffer containing 50 mM Tris-HCl, 250 mM sucrose, 5 mM $MgCl_2$, 2.5 KCl , 1 mM PMSF, 1 mM benzamidine-HCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.0044 TIU/ml aprotinin. One volume of nuclei isolation buffer containing 2.2 M sucrose was added and the resulting suspension was overlayed over 5 ml of this 2.2 M sucrose containing buffer. Nuclei pellets were pelleted by centrifugation at 70 900 $g_{av}$ for 60 min. Pellets were suspended in nuclei isolation buffer and one volume of 2.2 M sucrose containing nuclei isolation buffer was added. Finally the nuclei suspension was overlayed on 5 ml of 2.2 M sucrose containing nuclei isolation buffer and centrifugation was repeated. The pellet containing purified nuclei, was resuspended in 1 ml/g of nuclei isolation buffer.

### *Insulin Binding to Isolated Nuclei*

Insulin binding assays (34,35) were done on freshly isolated nuclei preparations. Insulin binding activity of nuclei was measured by incubating preparations containing 200 μg protein for 2 hours at $25^0$C with 4 nM of $^{125}$I-labeled porcine insulin (40 μCi/ug) (NEN Life Science Products, Boston, MA, U.S.A.). Samples were incubated in a final volume of 200 μl of buffer containing 62.5 mM sucrose, 5 mM Tris-HCl, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, pH 7.5, in the presence (20 μg/ml) and absence of excess unlabelled insulin. The receptor-$^{125}$I-labeled insulin complex was separated from free $^{125}$I-insulin by adding 0.6 ml 50 mM N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 0.1 % w/v rabbit γ-globulin, 10 % w/v polyethylene glycol 8000, and incubating for 15 min on ice before centrifugation at 14 000 $g_{max}$ for 5 min. The supernatant containing unbound insulin was aspirated and the pellets counted using Gamma 8000 counter (Beckman). The radioactivity precipitated in the presence of unlabelled insulin was considered non-specific binding. Specific binding was calculated by subtracting non-specific binding from the radioactivity precipitated in the absence of unlabelled insulin, and was expressed as fmol of insulin bound per mg protein.

### *Glucose and Insulin Determination*

Immediately after blood collection, serum was prepared by centrifugation at 2 000 $g_{max}$ for 10 min using a JS-4.3 rotor (Beckman Instruments). Serum was frozen at -$70^0$C until analysis. Serum glucose was determined using Glucose Trinder Reagent (Sigma Diagnostics, St. Louis, MO, U.S.A.) for the enzymatic determination of glucose. Insulin was measured using a commercial double antibody immunoassay for rat insulin (Linko Research, St. Louis, MO, U.S.A.).

### *Nuclei Purity and Protein Determination.*

Protein content of nuclei was determined (36). Nuclei were assayed for 5' nucleotidase activity as a measure of plasma membrane contamination (37). Contamination of isolated nuclei by plasma membrane was assessed by calculating the relative specific activity of the respective enzymes. Relative specific activity can be estimated by dividing the specific activity in units/min/mg protein of the marker enzyme in the nuclear fraction by the specific activity of the marker enzyme of the purified fraction where it is localized. This value is expressed as a percentage. Nuclei were analysed for DNA content (40). This value was estimated by dividing the amount in mg of DNA measured in purified nuclei by the amount of DNA measured in whole homogenate in mg and expressed as a percentage. Nuclei suspensions were inspected visually under the light microscope after staining with methylene blue (0.2% v/v) and ethidium bromide (100 µg/ml) at 400 x magnification.

### *Immunoblotting*

Samples of rat liver plasma membrane (100 µg protein), and nuclei (30 µg protein) were subjected to SDS-PAGE using a 7.5% (w/v) gel under reducing conditions (41), and electrotransferred to nitrocellulose. Nitrocellulose sheets were blocked for 1 hour at 25°C with 5% (w/v) bovine serum albumin Fraction V (Sigma) for analysis using anti-IR. Nitrocellulose sheets were then probed with anti-IR (1/25 dilution) overnight at 4°C in blocking solution containing 0.1% (v/v) Tween 20 and then washed with PBS, 0.1% (v/v) Tween 20. Detection was with horseradish peroxidase-conjugated secondary antibody (1/3000 dilution) followed by enhanced chemiluminesence imaging on X-ray film using ECL reagent (Amersham, Little Chafont Buckinghamshire, England). The intensity of the bands was quantified by scanning densitometry using the GS-670 Imaging Densitomer (BioRad Laboratories, Hercules, CA, USA).

***Purification of the insulin receptor***

Insulin receptor was purified from plasma membrane isolated from liver of male Sprague Dawley rats. Rats were housed and fed under the conditions given for mice. The University of Alberta Animal Welfare Committee approved the protocol. Rats were fasted for 12 hours prior to extraction of liver. To collect liver tissue, rats were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation. At time of sacrifice animals were eleven weeks of age, weighed 440 ± 17 g and liver tissue weighed 18.9 ± 0.7 g.

Plasma membrane was prepared according to an established procedure (42) modified as follows. Following sacrifice, livers were processed as described above. The post-nuclear supernatant was centrifuged at 8700 x $g_{max}$ for 20 min to pellet mitochondria. The remaining supernatent was spun at 105 000 x $g_{max}$ for 30 minutes in a Ti45 rotor to pellet plasma membranes. Plasma membranes were then purified by resuspending the pellet in buffer containing 0.25 M sucrose, 5 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM PMSF, 1 benzamidine-HCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin A pH 7.5, and then adding this buffer (1.04 volumes) containing 2.4 M sucrose so that the final concentration of the homogenate was 1.35 M. The suspension was aliquoted into centrifuge tubes and overlayered with 0.3 volumes of the 0.25 M sucrose-containing buffer. Tubes were centrifuged at 231 000 $g_{av}$ for 30 min using a T170.1 rotor (Beckman), and plasma membranes were collected at the interface.

Insulin receptor was purified from plasma membrane as described (34,43) with modifications. Plasma membranes were solubilized in a buffer containing 50 mM Tris, 1 mM PMSF, 1 mM benzamidine-HCl, 1 μg/ml leupeptin, 100 μg/ml pepstatin A, 10 TIU/ml aprotinin, and 2 % (v/v) Triton X-100 (Sigma, St. Louis, Missouri, USA) for two h at $4^0$C. Insoluble proteins were precipitated by centrifugation at 100 000 x $g_{av}$ for 1 hr in a SW28 rotor (Beckman). Supernatant was supplemented with 0.09 % (w/v) $MgCl_2$ and applied to *Triticum vulgaris* (Wheat germ)-agarose (Sigma) preequilibrated in 50 mM N-[Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) pH 7.6 , 1 mM PMSF, 1 mM benzamidine-HCl, and 150 mM NaCl, and mixed gently overnight at $4^0$C. The gel slurry was washed with 50 mM HEPES, 1 mM PMSF, 1 mM benzamidine, 150 mM NaCl, 10 mM $MgSO_4$, 0.1 % (v/v) Triton X-100, pH 6.0 (20 volumes), and 50 mM HEPES pH 7.6, 1 mM PMSF, 1 mM benzamidine-HCl, 150 mM NaCl, 0.1 % (v/v) Triton X-100, pH 7.6 (10 volumes). Insulin receptor bound to the column was eluted with 50 mM HEPES, 1 mM PMSF, 1 mM benzamidine-HCl,, 150 mM NaCl, 0.1 % (v/v) Triton X-100, 0.5 TIU/ml aprotinin, 20 μM pepstatin A, 20 μM leupeptin, and 0.3 M N-acetyl-D-glucosamine, pH 7.6. The eluted glycoprotein fraction was supplemented with 0.09 % (w/v) $MgCl_2$ and mixed overnight at $4^0C$ with Insulin-Agarose (Sigma) that had been preequilibrated with 50 mMTris-HCl, 0.1 mM PMSF, 0.1 % (v/v) Triton X-100 pH 7.5. The gel slurry was poured into a glass column and washed with 50 mM Tris-HCl, 0.1 mM PMSF, 0.1 % (v/v) Triton X-100, and 1 M NaCl, pH 7.5 (100 volumes). Insulin receptor bound to the column was eluted with 0.05 M sodium acetate, pH 5.0 (2 volumes). Fractions (1 ml) were collected into tubes containing 0.1 ml 1 M HEPES, 0.1 % (v/v) Triton X-100, 0.1 mM PMSF, and 20 mM ethyleneglycoltetraacetate, pH 7.8. Insulin receptor was assayed during various steps in the purification using insulin binding assays (**Appendix I**) and by iodination (**Appendix II**).

### *Insulin Receptor Antibody Preparation*

Purified insulin receptor was confirmed by mobility under reducing conditions on sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by insulin binding activity (34,35). Polyclonal antibody to purified insulin receptor was raised using standard techniques (44) in a Flemish Giant X Dutch Lop Ear rabbit housed at the Biological Sciences Center at the University of Alberta. Insulin receptor was injected subscapularly, using RIBI Adjuvant System (Cedar Lane, Hamilton, Montana, USA). Ten days following each inoculum, an enzyme-linked immunoassay (44) was used to test for the presence of antibodies in rabbit serum against insulin receptor in plasma membrane isolated from rat liver (**Appendix III, IV**). After four immunizations the rabbit was terminated and blood removed by cardiac puncture. Antibody to the β-subunit of the insulin receptor was purified from serum by incubation of the serum with the 95K

Da protein blotted onto nitrocellulose paper. After extensive washing with PBS, 0.1% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20), pH 7.3, anti-insulin receptor was eluted by a 2 min wash with 0.2 M glycine-HCl pH 2.8. Purified antibody, referred to as anti-IR, was tested for effectiveness in various immunoassays and was found to detect commercial and laboratory produced insulin receptor preparations in western blotting, and to immunoprecipitate insulin receptor in plasma membrane and nuclei preparations as effectively as commercially obtained antibodies including anti-human IR $\alpha$ subunit GRO7 from Oncogene Science Co. (Uniondale, NY, USA), or anti-human $\beta$-subunit from Transduction Laboratories (Lexington, Kentucky, USA) **(Appendix V)**.

### *Statistical Analysis*

The difference between time treatments following oral glucose gavage were assessed using analysis of variance procedures (SAS Version 6.11 SAS Inc., Cary, NC, USA) (45). For each of the seven timepoints 6 animals were analysed. A significant difference between treatments was determined by a Duncan's multiple range test ($p < 0.05$) (46)

## *RESULTS*

### *Animal Characteristics*

Body weights and liver weights of lean mice were similar among animals treated for different time periods following oral glucose gavage, and averaged 28.4 ± 0.3 g, and 1.29 ± 0.02 g respectively. Obese mice weighed 46.6 g ± 1.08 g and had liver weights of 1.84 ± 0.04 which was significantly greater than that of lean animals ($p<0.05$). There were no differences in body weights or liver weights between obese mice treated for different time periods following oral glucose gavage.

### *Nuclei Purity*

Contamination of purified nuclei was assessed in experiments done in **Chapter II**. The relative specific activity of nuclear membrane 5'nucleotidase, a plasma membrane marker, compared to the activity found in the plasma membrane averaged 3.18 ± 1.2 %. Inspection by bright field microscopy revealed intact nuclei with no contamination by cellular debris (**Chapter II, Figure 2.1**).

### *Serum Glucose and Insulin Concentrations*

Serum glucose concentrations in both lean and obese animals responded to oral glucose by increasing from concentrations at basal, 8.63 ± 1.46 mM and 13.58 ± 1.86 mM for lean and obese respectively, to 24.70 ± 1.32 mM and 33.66 ± 2.00 mM for lean and obese respectively, within 15 min following gavage (**Figure 3.1**). Within the time period tested, glucose levels were highest between 15 to 30 min following oral glucose in both lean and obese mice. At 120 and 180 min following oral glucose gavage, serum glucose concentrations decreased to basal levels in both lean and obese mice. Obese mice exhibited greater serum glucose concentration compared to lean mice following the 12 h fast and at 15, 30, 60, 90 min following oral glucose gavage ($p<0.05$).

Basal serum insulin concentrations in lean and obese mice were 19.00 ± 4.67 pM and 170 ± 16.65 pM, respectively. Within the time period tested, serum insulin concentrations peaked in both lean and obese mice within 15 to 30 min following oral glucose gavage **(Figure 3.2)** ($p<0.05$). By 30 min following the oral glucose meal, serum insulin concentrations in both lean and obese animals had fallen to those levels exhibited at fasting conditions. Obese mice exhibited hyperinsulinemia at fasting conditions and at all times selected following oral glucose gavage ($p<0.05$).

### *Insulin Binding to Isolated Nuclei*

Specific insulin binding to hepatocyte nuclei increased in lean animals within 15 min and then fell to values similar to basal by 60 min following the oral glucose meal **(Figure 3.3)**. Insulin binding to hepatocyte nuclei taken from obese animals did not appear to change following oral glucose gavage. The levels of insulin bound to hepatocyte nuclei taken from obese animals did not differ from the levels observed in lean animals. The effect of serum insulin on levels of nuclear insulin binding observed in lean versus obese animals was evaluated by plotting for each animal the amount of nuclear insulin binding versus serum insulin concentration **(Figure 3.4)**. As the amount of serum insulin increased following the glucose meal, a corresponding increase in insulin binding occurred in lean animals ($r^2 = 0.44$, $p< 0.01$), but not in obese animals ($r^2 = -0.06$, NS). This finding indicates that nuclear insulin receptor responsiveness to ligand is greater in lean animals compared to obese animals.

### *Immunoassay of insulin receptor in plasma membrane and isolated nuclei*

Insulin receptor levels were measured in hepatocyte plasma membrane and nuclei by western blotting analysis using an anti-insulin receptor antibody. Only times 0, 15 and

180 min following oral glucose gavage were assessed (**Figure 3.5**). Plasma membrane levels of insulin receptor were significantly reduced from basal levels at 180 min following oral glucose treatment. The level of insulin receptor in plasma membrane lean and obese animals was similar at basal and at all timepoints following oral glucose.

Insulin receptor levels in the nuclei of obese but not lean mice were increased from fasting conditions at 15 and 180 min following oral glucose gavage (**Figure 3.6**). At 180 min following the oral glucose meal insulin receptor levels in nuclei remained high. The levels of nuclear insulin receptor found in the nuclei were similar at basal conditions but the change from basal was greater for obese animals compared to lean animals at 15 and 180 min following oral glucose.

## *DISCUSSION*

In the present study eight week old C57BL/6J-ob/ob mice presented with high blood glucose and high plasma insulin levels at both fasting conditions and following an oral glucose bolus. Obese mice of this strain exhibit diabetic syndromes after four weeks of age and this animal model is used to study the pathogenesis of obesity and diabetes mellitus in humans (45). In both lean and obese mice, serum glucose levels peaked within 15 to 30 min following oral glucose gavage. Changes in serum glucose following oral glucose administration were paralleled by changes in serum insulin which peaked within 15 to 30 min in both lean and obese animals. The rise in serum insulin concentration that occurred in response to oral glucose in the obese mice indicates that at eight weeks these mice have not lost the capacity to produce and secrete insulin, which occurs with the development of overt diabetes (45).

There was no significant change in insulin receptor concentration in the plasma membrane at 15 min following oral glucose in lean or obese mice. Plasma membrane insulin receptor levels were significantly reduced compared to basal levels by 26 % in lean and 41 % in obese mice at 180 min following an oral glucose meal. Previous studies have reported that stimulation with insulin reduces the number of cell surface insulin receptors due to ligand-induced internalization (25,46-49). The rate of loss of receptors from the plasma membrane was reported to be 30 % within 15 min following insulin stimulation in one study using adipocytes (46). In other studies (25,46-49), the percentage of total cell-associated insulin receptor internalized was reported. As the plasma membrane pool represents 90 % of the total amount of cell-associated receptor, the amount of plasma membrane insulin receptor internalized may be estimated to closely approximate the decrease in cell-associated receptor (46,48). Thus the rate of loss of receptors from the plasma membrane can be estimated to be 27 to 36 % in 15 min. These studies report more rapid rates of insulin receptor internalization than what was observed in the present study, which may reflect the differences in methodologies used to measure insulin receptor concentration. In the above studies (25,46-49), insulin receptor levels were measured using [$^{125}$I]-labeled insulin binding assay which estimates the insulin binding capacity of the plasma membrane compartment. In the present study, insulin receptor levels were estimated by western blotting. The immunoassay may have higher sensitivity than [$^{125}$I]-labeled insulin binding assay. The use of a polyclonal antibody preparation involves multiple antibody molecules binding to a given polypeptide, which may not be in its native form (50). A limitation of the insulin binding assay, when used to measure receptor concentrations, is that ligand-antibody recognition may not occur if the receptor is not in its native conformation (51).

Obesity was not found to affect the rate of ligand-induced internalization of the insulin receptor in the present study. A reduced amount of insulin binding to plasma membrane observed in obesity is speculated to be due to impaired internalization and processing of receptor following ligand binding. A reduced amount of insulin-induced receptor internalization has been reported to occur in obesity (8,25,26,52) in some but not all studies (27). In one study (27), the defect in obesity occurred not during internalization but during the processing and recycling of the receptor back to the plasma membrane. This suggests that decrease in insulin receptor binding observed in the plasma membrane obesity may occur at a step subsequent to insulin receptor internalization.

Insulin binding to hepatocyte nuclei was increased in lean mice within 15 to 30 min following oral glucose gavage. This peak in insulin binding to the nucleus was within the time course of the increase in serum insulin concentration. Insulin receptor levels in the nucleus were not increased by an oral glucose meal when measured by immunoassay using anti-insulin receptor antibody. One explanation for this is that small changes in nuclear receptor levels that are not measurable by immunoassay, as there is a basal amount of insulin receptor observed in the nucleus (31). Changes in nuclear insulin binding following oral glucose gavage were not demonstrated in obese animals. When nuclear insulin binding was normalized to the amount of insulin present in the serum of both lean and obese mice, obese mice appeared more resistant to insulin-induced translocation to the nucleus. However western-blotting assay detected a 250 % increase in insulin receptor concentration compared to basal levels in the nuclei of obese animals within 15 min following a meal of glucose. The change in nuclear insulin receptor concentration in obese hepatocytes was higher than that in lean animals following oral glucose gavage. These findings suggest that in obese compared to lean mice, insulin receptor translocation is enhanced, possibly due to hyperinsulinemia. The lack of a change in insulin binding to the nucleus of obese mice hepatocytes may indicate that the nuclear insulin receptor in obese animals has reduced ligand affinity.

In conclusion, insulin receptor translocation to the nucleus was demonstrated in both lean and obese animals within 15 min following a meal of glucose. If ligand-induced insulin receptor translocation to the nucleus plays a role in insulin signaling to the nucleus, alterations in translocation rates of the insulin receptor induced by obesity may be responsible for the changes in some of the insulin-responsive gene expression that have been demonstrated in obesity (53,54)

## *REFERENCES*

1) Golay, A., and Felber, J.P. Evolution from obesity to diabetes. (1994) Diabetes Metab.,20:3-14.

2) Smith, S.R. The endocrinology of obesity. (1996) Endocrinol.,25(4):921-942.

3) Garvey, W.T., and Birnbaum, M.J. Cellular insulin action and insulin resistance. (1993) Bailliere's Clinical Endocrinol. Metab.,7(4):785-873.

4) Goodyear, L.J., Giorgino, F., Sherman, L.A., Carey, J., Smith. R.J., and Dohm, G.L. Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation and phosphatidyl 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. (1995) J. Clin. Invest.,95(5):2195-1204.

5) Pederson, O. Studies of insulin receptor binding and insulin action in humans. (1984) Danish Med. Bull.,31:1-31.

6) Adamo, M., Shemer, J., Arider, M., Dixon, J., Carswell, N., Bhathena, S.J., and Michaelis, O.E. 4th. Liver insulin receptor tyrosine kinase activity in a rat model of type II diabetes mellitus and obesity. (1989) J. Nutr.,119(3):484-489.

7) Meier, D.A., Hennes, M.M., McCune, S.M., and Kissebah, A.H. Effect of obesity and gender on insulin receptor expression in liver of SHHF/Mac-FAcp rats. (1995) Obesity Res.,3(5):465-470.

8) Poole, G.P., Pogson, C.I., O'Conner, K.J., and Lazarus, N.R. The metabolism of 125-I-labeled insulin by isolated Zucker rat hepatocytes. (1981) Bioscience Reports,1(12):903-910.

9) Svedberg, J., Bjorntorp, P., Smith, U., and Lonnroth, P. Effect of free fatty acids on insulin receptor binding and tyrosine kinase activity in hepatocytes isolated from lean and obese rats. (1992) Diabetes,41(3):294-298.

10) Bjorntorp, P., Berchtold, P., and Tibblin, G. Insulin secretion in relation to adipose tissue in man. (1971) Diabetes,20:65-70.

11) Hennes, M.M., Shrago, E., and Kissebah, A.H. Receptor and post-receptor effects of free fatty acids (FFA) on hepatocyte insulin dynamics. (1990) Int. J. Obesity,14(10):831-841.

12) Olefsky, J.M., and Reaven, G.M. (1977) Diabetes,26:680-.

13) Gavin, J.R. III, Roth, J.R., Neville, D.M. Jr., DeMeyts, P., and Duell, D.N. (1974) Proc. Natl. Acad. Sci. USA,71:84-.

14) Kobayashi, M., and Olefsky, J.M. (1978) Am. J. Physiol.,235:953-.

15) Caro, J.F., Ittoop, O., Pories, W.J., Meelheim, D., Flickinger, E.G., Thomas, F., Jenquin., M., Silverman., J.F., Khazanie, P.G., and Singa, M.K. Studies on the mechanism of insulin resistance in the liver from humans with non-insulin dependant diabetes mellitus. (1986) J. Clin. Invest.,78:249-258.

16) Friedenberg, G.R., Henry, R.R., Klein, H.H., Reichart, D.R., and Olefsky, J.M. Decreased kinase activity of insulin receptors from adipocytes of non-insulin dependent diabetic subjects. (1987) J. Clin. Invest.,79:240-250.

17) Hurrell, D.G., Pederson, O., and Kahn, C.R. Alteration in hepatic insulin receptor kinase in genetic and acquired obesity in rats. (1989) Endocrinol.,125(5):2454-2462.

18) LeMarchand-Brustel, Y., Gremeaux, T., Ballotti, R., and Van Obberghen, E. Insulin receptor tyrosine kinase is defective in skeletal muscle of insulin-resistant obese mice. (1985) Nature,315:676-678.

19) Saad, M.J.A., Araki, E.M., Miralpeix, M., Rothenberg, P.L., White, M.F., and Kahn, C.R. Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. (1992) J. Clin. Invest.,90:1839-1849.

20) Sredy, J., Sawicki, D.R., Flam, B.R., and Sullivan, D. Insulin resistance is associated with abnormal dephosphorylation of a synthetic phosphopeptide corresponding to the major autophosphorylation site of the insulin receptor. (1995) Metab. Clin. Exper.,44(8):1074-1082.Takayama, S., White, M.F., and

21) Hotamisligil, G.S., Peraldi, P., Budavari, A., Ellis, R., White, M.F. and Spiegelman, B.M. IRS-1-mediated inhibition of insulin-receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. (1996) Science,271:665-668.

22) Kahn, C.R. Phorbol ester-induced serine phosphorylation of the insulin receptor decreases its tyrosine kinase activity. (1988) J. Biochem.,263(7):3440-3447.

23) Kablaoui, B., Jongsoon, L., and Pilch, P.F. Dynamics of signaling during insulin-stimulated endocytosis of its receptor in adipocytes. (1995) J. Biol. Chem.,270(1):59-65.

24) Khan, M.N., Baquiran, G., Brule, C., Burgess, J., Foster, B., Bergeron, J.J.M., and Posner, B.I. Internalization and activation of the rat liver insulin receptor kinase *in vivo*. (1989) J. Biol. Chem.,264(22):12931-12940.

25) Trischitta, V., Brunetti, A., Chiavetta, A., Benzi, L., Papa, V., and Vigneri, R. Defects in insulin-receptor internalization and processing in monocytes of obese subjects and obese NIDDM patients. (1989) Diab.,38:1577-1584.

26) Grunberger, G., Gerger, D., Carpentier, J.L., Robert, A., and Gorden, P. Receptor-mediated endocytosis of insulin: inhibition of [125I]iodo-insulin internalization in insulin-resistant diabetic states of man. (1989) Acta Endocrinol.,121(4):581-586.

27) Sesti, G., D'Alfonso, R., Vargas Punti, M.D., Tullio, A.N., Liu, Y.Y., Federici, M., Borboni, P., Marini, M.A., Lauro, R., and Fusco, A. Delayed intracellular dissociation of the insulin-receptor complex impairs receptor processing in cultured Epstein-Barr virus-transformed lymphocytes from insulin-resistant subjects. (1996) Diabetologia,39(3):289-295.

28) Podlecki, D.A., Smith, R.M., Kao, M., Tsai, P., Huecksteadt, T., Brandenburg, D., Sasher, R.S., Jarett, L. and Olefsky, J.M. Nuclear translocation of the insulin receptor. A possible mediator of insulin's long term effects. (1987) J. Biol. Chem.,292:3362-3363.

29) Wong, K.Y., Hawley, D., Vigneri, R., and Goldfine, I.D. Comparison of solubilized and purified plasma membrane and nuclear insulin receptors. (1988) Biochem.,27:375-379.

30) Smith, R.M., and Jarett, L. Ultrastructural evidence for the accumulation of insulin in nuclei of intact 3T3-L1 adipocytes by an insulin receptor-mediated process. (1987) Proc. Natl. Acad. Sci. USA, 84:459-463.

31) Kim, S-J., and Kahn, C.R. Insulin induces rapid accumulation of insulin receptors and increases tyrosine kinase activity in the nucleus of cultured adipocytes. (1993) J. Cell. Physiol.,157:217-228.

32) Cheema, S.K., Venkatramen, J., and Clandinin, M.T. Insulin binding to liver nuclei from lean and obese mice is altered by diet fat. (1992) Biochim., Biophys. Acta,1117:37-41.

33) Blobel, G., and Potter, V.R. Nuclei from rat liver: isolation method that combines purity wth high yield. (1966) Science, 154:1662-1665.

34) Fujita-Yamaguchi, Y., Choi, S., sakamoto, Y., and Itakura, K. Purification of insulin receptor with full binding activity. (1983) J. Biol. Chem.,258(8):5045-5049.

35) Desbuquois, B., and Aurbach, G.D. Use of polyethylene glycol to separate free and antibody bound peptide hormones in radioimmunoassays. (1971) J. Clin. Endocrinol.,33:732-738.

36) Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the Folin phenol reagent. (1951) J. Biol. Chem., 193:265-275.

37) Aronson, N.N.Jr., and Touster, O. Isolation of rat liver plasma membrane fragments in isotonic sucrose. (1974) Methods in Enzymology, 31:90-102.

38) Green, D.E., Mii, S., and Kohout, P.M. Studies on the terminal electron transport system. 1) Succinate dehydrogenase (1955) J., Biol., Chem., 551-567.

39) Swanson, M.A. (1955) Meth. Enzymol.,2:541-543

40) Burton, B.J.(1956) Biochem. J., 62:315-318.

41) Laemmli, U.K. (1970) Nature (London),227:680-685.

42) Fleischer, S., and Kervina, M. Subcellular fractionation of rat liver. (1974) Meth. Enzymol.,31:6-41.

43) Helmerhorst, E., and Nakhoul, A.-M. Novel affinity support prepared by crosslinking purified insulin receptors to agarose. (1993) Biochem. Biophys. Res. Comm., 195(1):237-241.

44) Assil, H.I., Sheth, H., and Sporns, P. An ELISA for sulfonamide detectioin using affinity-purified polyclonal antibodies. (1992) Food Res. Int., 25:343-353.

45) Herberg, L., and Coleman, D.L. Laboratory animals exhibiting obesity and diabetic syndromes (1977) Metabolism, 26(1):59-99.

46) Marshall, S. Kinetics of insulin receptor internalization and recycling in adipocytes. (1985) J. Biol. Chem.,260(7):4136-4144.

47) Backer, J.M., Shoelson, S.E., Haring, E., and White, M.F. Insulin receptors internalize by a rapid, saturable pathway requiring receptor autophosphorylation and an intact juxtamembrane region. (1991) J. Cell Biol.,115(6):1535-1545.

48) Backer, J.M., Kahn, C.R., and White, M.F. Tyrosine phosphorylation of the insulin receptor during insulin-stimulated internalization in rat hepatoma cells. (1989) J. Biol. Chem.,264(3):1694-1701.

49) Shranz, D.B., Rohilla, A.M.K., Anderson, C., Wood, W.M., and Berhanu, P. Insulin internalization in the absence of the insulin receptor tyrosine kinase domain is insufficient for mediating intracellular biological effects. (1996) Biochem. Biophys. Res. Comm.,227(2):600-607.

50) Harlow, E., and Lane, D.,(Eds) Immunoblotting. In: Antibodies, a laboratory manual. (1988) Cold Spring Harbour, New York, USA, pp471-510.

51) Gammeltoft, S. Insulin receptors: binding kinetics and structure function relationships to insulin. (1984) Physiol. Rev.,64(4):1321-1379.

52) Trischitta, V., Gullo, D., Squatrito, S., Pezzino, V., Goldfine, I.D., and Vigneri, R. Insulin internalization into monocytes is decreased in patients with type II diabetes mellitus. (1986) J. Clin. Endocrinol. Metab.,62(3):522-528.

53) Iritani, N., Hosomi, H., Fukuda, H., and Ikeda, H. Polyunsaturated fatty acid regulation of lipogenic enzyme gene expression in liver of genetically obese rats. (1995) Biochim. Biophys. Acta,1255:1-8.

54) Dugail, I., Quignard-Boulange, A., Le Liepvre, X., and Ardouin, B. Gene expression of lipid storage-related enzymes in adipose tissue of the genetically obese Zucker rat: coordinate increase in transcriptional activity and potentiation by hyperinsulinemia. (1992) Biochem. J.,281(3):607-611.

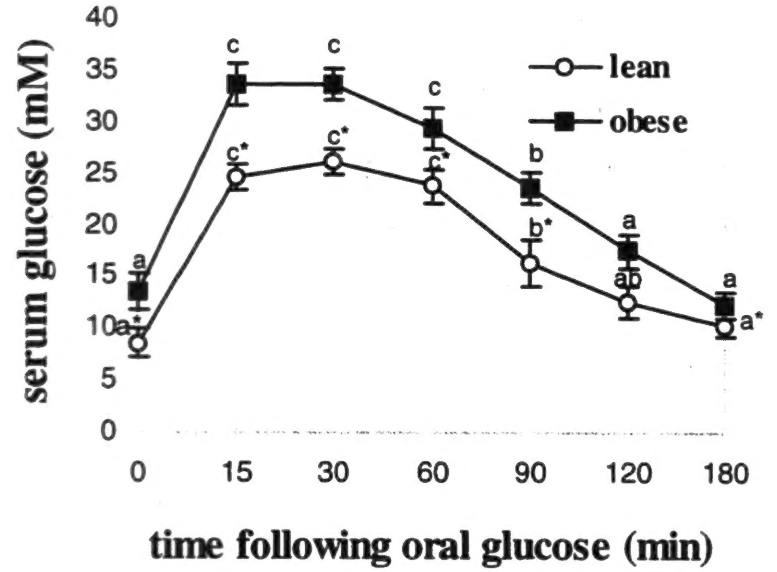


**Figure 3.1 Serum glucose response to oral glucose gavage in eight week lean and obese mice.** Mice were fasted for 12 hours before treatment with 3 g/kg body weight glucose. After selected time periods following treatment, mice were anesthetized and blood removed by cardiac puncture. Serum was prepared and assayed for glucose which was expressed in mM. Data represents mean ± standard error of the mean (n = 6 per timepoint). Values with different letters within the same curve are significantly different. ($p<0.05$). A value labeled with (*) within the lean curve is significantly different from the obese value at the same timepoint ($p<0.05$).

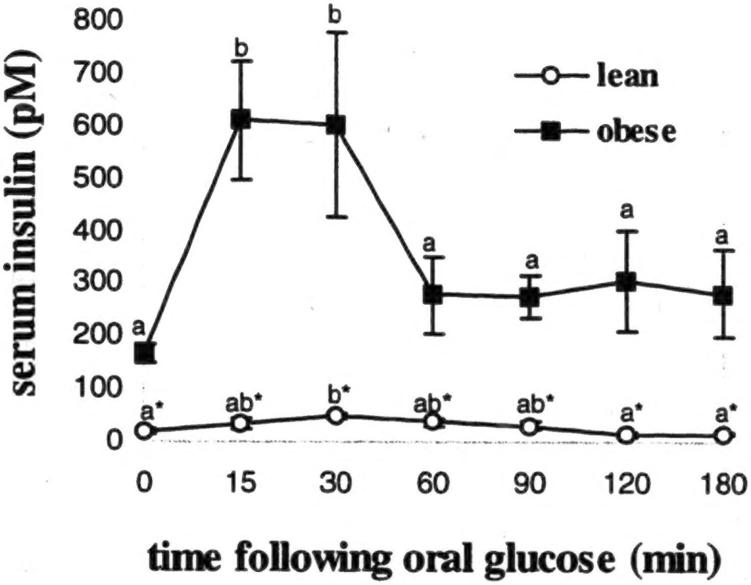


**Figure 3.2 Serum insulin response to oral glucose gavage in eight week lean and obese mice.** Mice were fasted for 12 hours before treatment with 3 g/kg body weight glucose. After selected time periods following treatment, mice were anesthetized and blood removed by cardiac puncture. Serum was prepared and assayed for insulin which was expressed in pM. Data represents mean ± standard error (n = 6 per timepoint). For comparisons between basal timepoint (0 min) and other times following oral glucose gavage of serum insulin of lean animals, statistical difference was determined by Students 's *t* test. Other comparisons are made using analysis of variance. Values with different letters within the same curve are significantly different ($p<0.05$). A value labeled with (*) within the lean curve is significantly different from the obese value at the same timepoint ($p<0.05$).

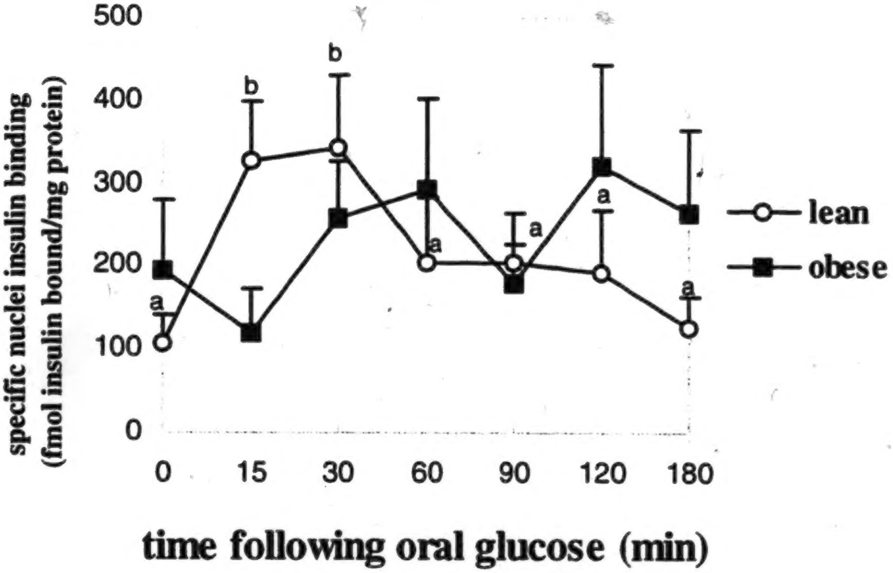


**Figure 3.3 The response of [$^{125}$-I]-labeled insulin binding to isolated hepatocyte nuclei of lean and obese mice following oral glucose gavage.** Mice were fasted for 12 hours before treatment with 3 g/kg body weight glucose. After selected time periods following treatment, liver was removed and nuclei isolated. 200 ug of nuclei extract was used in an insulin binding assay. Specific insulin binding was measured in fmol insulin bound per mg protein. Data represents mean ± standard error (n = 6 per timepoint). Statistical difference was determined by Student's *t* test. Comparisons were made between selected time following glucose gavage and basal condition (0 min). Values with different letters within the same curve are significantly different ($p<0.05$). Days were grouped into block and block was tested for significance using ANOVA. There was no significant difference between days.

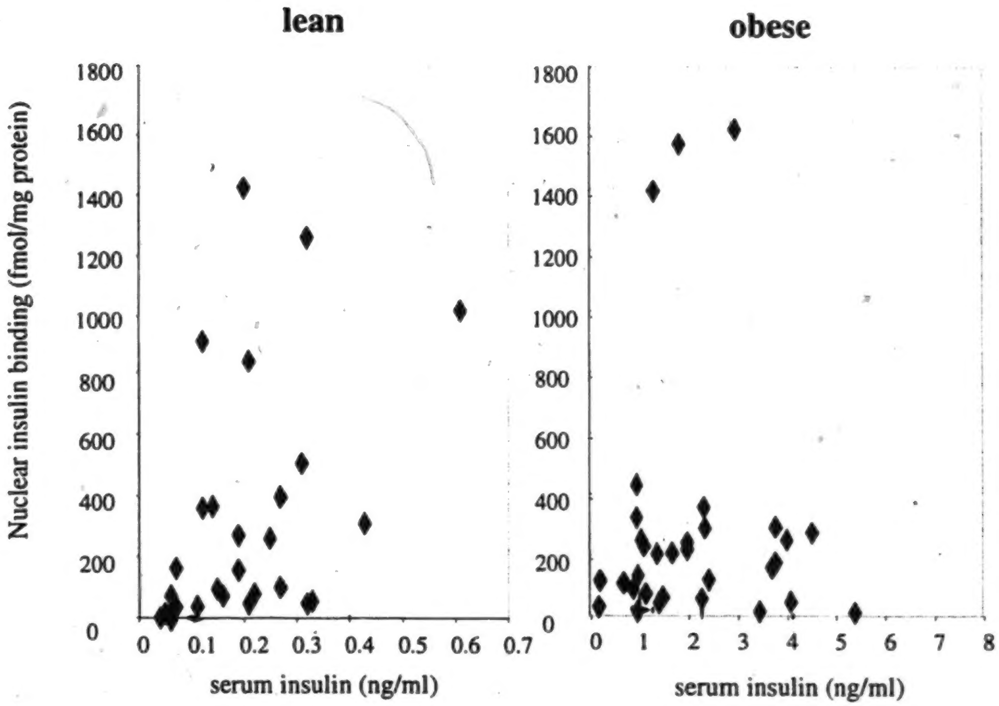


**Figure 3.4 Specific insulin binding to hepatocyte nuclei of lean and obese mice following oral glucose gavage versus serum insulin.** Mice were fasted for 12 hours before treatment with 3 g/kg body weight glucose. After selected time periods following treatment, livers were removed and nuclei isolated. 200 ug of nuclear extract was used in an insulin binding assay. For each animal specific insulin binding was measured in fmol insulin bound per mg protein plotted versus serum insulin concentration in ng/ml. (n = 6 per timepoint). Pearson correlation coefficients for lean animals $r^2 = 0.44$, $p < 0.01$ and for obese animals $r^2 = -0.06$, NS.

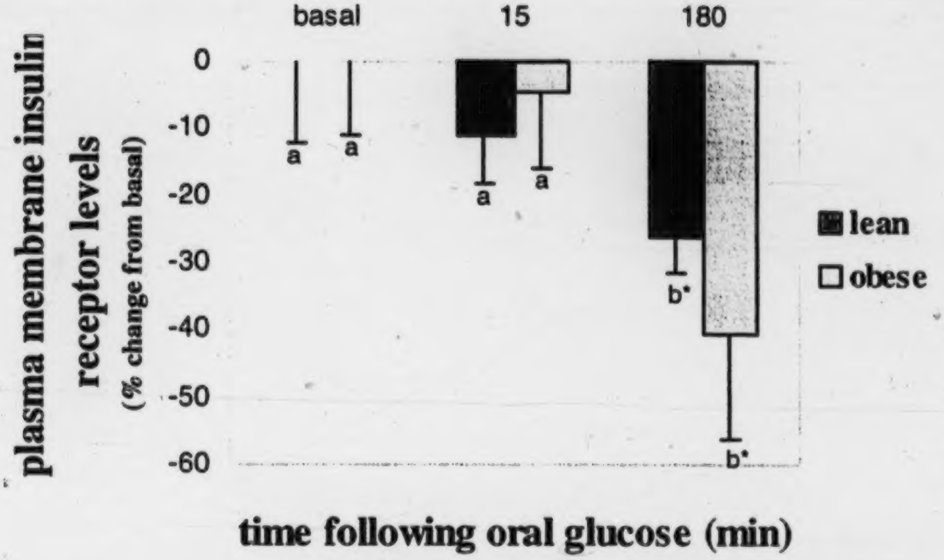


**Figure 3.5 Plasma membrane insulin receptor levels measured by western blotting immunoassay.** Mice were fasted for 12 hours before treatment with 3 g/kg body weight glucose. After selected time periods following treatment, liver was removed and nuclei isolated. Plasma membrane was purified and 100ug protein samples were subjected to SDS-PAGE followed by western blotting using anti-insulin receptor antibody. Values are expressed in densitometer units. Data represents mean ± standard error (n = 6 per timepoint). Statistical difference was determined by Student's *t* test. Comparisons were made between selected time following glucose gavage and basal condition (0 min) (0 timepoint = 1.94 ±0.24 units). Values with different letters within the same curve are significantly different ($p<0.05$).

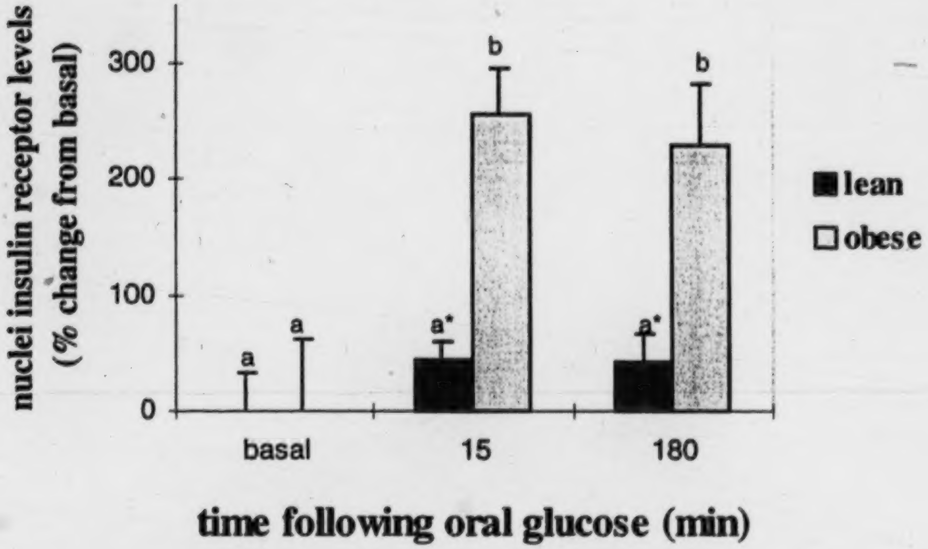


**Figure 3.6 Nuclear insulin receptor levels measured by western blotting immunoassay.** Mice were fasted for 12 hours before treatment with 3 g/kg body weight glucose. After selected time periods following treatment, liver was removed and nuclei isolated. 30 ug protein samples were subjected to SDS-PAGE followed by western blotting using anti-insulin receptor antibody. Values are expressed in densitometer units. Data represents mean ± standard error (n = 6 per timepoint). Statistical difference was determined by Student's *t* test. Comparisons were made between selected time following glucose gavage and basal condition (0 min =0.65 ±0.21 units). Values with different letters within the same curve are significantly different ($p<0.05$). A value labeled with (*) within the lean curve is significantly different from the obese value at the same timepoint ($p<0.05$).

# CHAPTER IV

# ORAL GLUCOSE-INDUCED INSULIN RECEPTOR TRANSLOCATION TO THE HEPATOCYTE CELL NUCLEUS IS COINCIDENT WITH DECREASES IN DNA BINDING PROTEIN PHOSPHORYLATION AND INCREASES IN GENE EXPRESSION

## *INTRODUCTION*

The insulin receptor resides in the plasma membrane with its $\alpha$ and $\beta$ subunits in a $\alpha_2\beta_2$ oligomeric conformation (1). Upon binding of insulin to the $\alpha$ subunit conformational changes occur that result in autophosphorylation of the $\beta$-subunit on specific tyrosine residues (2). Autophosphorylation causes an intrinsic tyrosine kinase within the $\beta$-subunit to become active toward intracellular substrates. Insulin binding and tyrosine kinase activity are well studied functions of the insulin receptor that are responsible for mediating insulin action much of within responsive cells (3,4).

There is increasing evidence for the ability of insulin to directly influence nuclear processes such as induction of transcription of the intermediate early genes c-fos, and c-jun, as well as various other metabolic genes including glyceraldehyde-6-phosphate dehydrogenase (GAPDH), malic enzyme (ME), and fatty acid synthase (5). Insulin stimulation of cells is also known to cause repression of transcription of phosphoenolpyruvate carboxykinase and efflux of mRNA from the nucleus (5). The mechanism responsible for transmission of the insulin signal to the nucleus of the cell is unknown. Once the plasma membrane receptor is activated by insulin binding, plasma membrane pools of activated receptor trigger a cascade of phosphorylation / dephosphorylation via intracellular kinases. These kinases, including insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3'-kinase (P-I-3'K), and mitogen activated protein kinase (MAP-K), transduce the insulin signal to the cell nucleus (3-7). However some studies have found that nuclear responsiveness to insulin is maintained in spite of selective inactivation of IRS-1 (8,9), S6 kinase (10), or phosphatidylinositol 3-kinase (11). This evidence suggests that an alternate mechanism for transduction of the insulin signal to the nucleus may occur.

Insulin binding to its receptor is followed by internalization of the insulin-receptor complex within seconds (12-14). Internalization has the effect of clearing insulin, down regulating the receptor, receptor recycling and possibly intracellular signaling. Following insulin binding ligand-receptor complexes have been localized to subcellular compartments such as endosomes (14), and to the nucleus (15). It has been shown that within the endosomal apparatus the insulin receptor is more highly autophosphorylated and may be at a more favored site to interact with intracellular substrates compared to the receptor residing on the plasma membrane (14). Translocation of activated insulin receptor from plasma membrane pools to the nucleus may provide an alternate and independent mechanism by which the insulin signal reaches the cell nucleus.

Studies have suggested that insulin receptors are present at the cell nucleus (15-22). Several investigators have shown the appearance in the nucleus of insulin receptors *after* insulin stimulation of the cell, using techniques such as ferritin- (15), radio- (16,17) or photoaffinity- (19) labeling of ligand as well as immunodetection of the receptor (18,20). Attempts have been made to determine the subcellular compartments through which the insulin receptor moves during recycling. Using photoactive insulin analogs that covalently attach to the insulin receptor, insulin-receptor complexes have been followed by electron microscopic autoradiography. These complexes have been found moving from the plasma membrane into endosomes, though lysosomes, and back though endosomes before moving back to the plasma membrane (23). One caveat of this method is that the covalent complex of insulin with the insulin receptor is unable to dissociate, therefore it is important to determine if the path taken by the receptor-ligand complex is that followed under normal conditions or that taken when ligand dissociation is prevented.

Translocation to the nucleus of other polypeptide hormone receptors including growth hormone receptor (24), epidermal growth factor receptor (25,26), prolactin receptor (27), fibroblast growth factor receptor (28) and nerve growth factor receptor (29) has been reported. The precise mechanism for nuclear accumulation of insulin receptor or for other growth factor receptors has not been elucidated, although the internalization of insulin receptors upon ligand binding and subsequent trafficking of receptors from the endosomal apparatus to intracellular organelles and recycling to the plasma membrane are documented (12-14). In **Chapter II** it was demonstrated that an oral glucose meal given to mice fasted for 12 h induced an increase in insulin binding to the hepatocyte nucleus.

The present study was designed to test the hypothesis that insulin stimulation *in vivo* results in translocation of active insulin receptor to the nucleus which transmits the insulin signal to the nucleus, resulting in transcription of insulin responsive genes involved in glucose and lipid metabolism. To test this hypothesis *in vivo*, mice were fasted for 24 h then given a meal of glucose to produce insulin stimulation. It was speculated that a longer fast would enhance responses to insulin following the glucose meal than that observed in **Chapter II**. Hepatocyte nuclei were isolated at selected timepoints following the glucose meal and were assayed for insulin receptor levels and insulin receptor autophosphorylation.

## *MATERIALS AND METHODS*

### ***Insulin Receptor Antibody Preparation***

Insulin receptor was purified from rat liver plasma membranes using established procedures (30,31). Receptor was confirmed by mobility on sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and by insulin binding activity (31,32). A Polyclonal antibody to purified insulin receptor was raised using standard techniques (33) in a Flemish Giant X Dutch Lop Ear rabbits, housed at the Biological Sciences Center at the University of Alberta. Insulin receptor was injected subscapularly using RIBI Adjuvant System (Ceder Lane, Hamilton, Montana, USA). Ten days following each inoculum, an enzyme-linked immunoassay (33) was used to test for the presence of antibodies in rabbit serum against insulin receptor in plasma membrane purified from rat liver. After four immunizations at 2 month intervals after the first booster, the rabbit was terminated and blood removed by cardiac puncture. Antibody to the β-subunit of the insulin receptor was purified from serum by incubation of the serum with the 95K Da insulin receptor subunit blotted onto nitrocellulose paper. After extensive washing with PBS containing 0.1% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20), pH 7.3, anti-insulin receptor was eluted by a 2 min wash with 0.2 M glycine-HCl pH 2.8. Purified antibody, referred to as anti-IR, was tested for effectiveness in various immunoassays and was found to detect commercial and laboratory produced insulin receptor preparations in western blotting, and to immunoprecipitate insulin receptor in plasma membrane and nuclei preparations as effectively as commercially obtained antibodies including anti-human IR α subunit GRO7 from Oncogene Science Co. (Uniondale, NY, USA), or anti-human β-subunit from Transduction Laboratories (Lexington, Kentucky, USA) (**Appendix II,III,IV,V**).

### *Animals Treatments*

Male C57 Black 6 mice (Jackson Laboratories, Bar Harbour, Maine, U.S.A.), were fed a standard laboratory chow (Richmond Laboratory Standard Rodent Diet 5001, PMI Feeds, Inc., St. Louis, MO, USA) *ad libitum*, from the age of weaning. Animals were housed in a temperature and humidity controlled room with a 12 hour light/dark cycle. Animals were housed in groups of four to six in polypropylene cages. The University of Alberta Animal Welfare Committee approved the protocol. At eight weeks of age, mice were fasted for 24 hours beginning at 9:00 am preceding the experimental day. Mice were then given an oral gavage of 3 g glucose/kg body weight. At various times following the gavage animals were anesthetized with halothane and then blood was removed by cardiac puncture for glucose and insulin determinations. Animals were terminated by cervical dislocation.

### *Nuclei Isolation*

The liver was immediately excised and minced in homogenization buffer containing 250 mM glucose, 50 mM Tris-HCl, 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl flouride (PMSF), 10 mM NaF, 10 mM $NaVO_3$, 10 mM $NaP_2O_5$, 1 mM $MgCl_2$, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 0.0044 Trypsin Inhibitory Units (TIU)/ml aprotinin at pH 7.5. Nuclei were isolated as described by Blobel and Potter (34), with the following modifications. Tissue was homogenized using a Potter-Evelhjem homogenizer with a motor driven teflon pestle and then centrifuged at 1000 x g for 20 min using a Beckman JA20 rotor (Paloalto, California, USA). Pellets were resuspended in homogenization buffer and then the sucrose concentration of the homogenate was raised to 1.6 M using homogenization buffer containing 2.4 M sucrose. The homogenate was centrifuged at 124 000 x g for 70 min using a Beckman SW 28 rotor. Using a homogenizer nuclei pellets were resuspended in nuclei isolation buffer containing 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2.5 mM KCl, 1 mM benzamidine-HCl, 1 mM PMSF, 2 mM NaF, 2 mM $NaVO_3$, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 0.0044 TIU/ml aprotinin. One volume of nuclei isolation buffer containing 2.2 M sucrose, was added to the nuclear suspension. This mixture was layered over five ml of the 2.2 M sucrose containing buffer and centrifuged at 124 000 x g for 60 min using a SW28 rotor. Nuclei pellets were resuspended in nuclei isolation buffer, mixed with one volume of 2.2 M sucrose containing buffer, layered over 2.2 M sucrose containing buffer and recentrifuged at 124

000 x g for 60 min. Pellets were resuspended using a 18 G needle in 0.5 ml nuclei isolation buffer, and 0.5 ml solubilization buffer containing 0.25 M sucrose, 100 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM benzamidine, 100 mM $NaP_2O_7$, 10 mM $NaVO_3$, 100 mM NaF, 2 % (v/v) Triton X-100 (Sigma, St. Louis, Missouri, USA), 10 μg/ml leupeptin, pH 7.5. DNase 1 and RNase A were added to these suspensions to final concentrations of 20 μg/ml. Samples were incubated overnight at $4^0$C with rotation and then centrifuged 14 000 x g for 30 min to collect soluble proteins referred to as nuclei extract.

***Purity of Nuclei and Protein Determination.***

Protein content of nuclear extracts was determined (35). Cell nuclear extracts were assayed for 5' nucleotidase activity as a measure of plasma membrane contamination (36), succinate cytochrome c reductase activity as a measure of mitochondrial contamination (37), and glucose-6-phosphatase activity as a measure of endoplasmic reticulum (38). Contamination of isolated nuclei by plasma membrane, mitochondria, or endoplasmic reticulum was assessed by calculating the relative specific activity of the respective enzymes. Relative specific activity can be estimated by dividing the specific activity in units/mg protein/min of the marker enzyme in the nuclear fraction by the specific activity of the marker enzyme of the purified fraction where it is localized. This value is expressed as a percentage. A relative specific activity of less than 5 % indicated nuclei purity (18,20). Nuclei were analysed for DNA content (39). This value was estimated by dividing the amount of DNA (mg) measured in purified nuclei by the amount of DNA measured in whole homogenate (mg) and expressed as a percentage.

Nuclear suspensions were inspected visually under the light microscope after staining with methylene blue (0.2% v/v) and ethidium bromide (100 μg/ml) at 400 x magnification.

### *Immunoblotting*

Samples of rat liver nuclei extract (100 μg protein) were precipitated by adding an equal volume of cold acetone, incubated at -20$^0$C and then centrifuged at 5 000 x g to pellet proteins. Samples were subjected to SDS-PAGE using a 7.5% (w/v) gel under reducing conditions (40), and electrotransferred to nitrocellulose. Nitrocellulose sheets were blocked for 1 hour at 25$^0$C with 5% (w/v) bovine serum albumin Fraction V (Sigma) for analysis using anti-IR or 3% (w/v) non fat skim milk in phosphate-buffered saline (PBS), pH 7.3 for analysis using anti-phosphotyrosine (anti-PY), (Upstate Biotechnology Inc., Lake Placid, New York, USA). Nitrocellulose sheets were then probed with anti-IR (1/25 dilution) or anti-PY (1 μg/ml) overnight at 4°C in blocking solution containing 0.1% (v/v) Tween 20 and then washed with PBS, 0.1% (v/v) Tween 20. Detection was with horseradish peroxidase-conjugated secondary antibody (1/3000 dilution) followed by enhanced chemiluminesce imaging on X-ray film using ECL reagent (Amersham, Little Chafont Buckinghamshire, England). The intensities of the bands were quantified by scanning densitometry using the GS-670 Imaging Densitomer (BioRad Laboratories, Hercules, CA, USA).

### *Immunoprecipitation*

Nuclear extract (300 μg protein) was precleared three times each with rabbit serum agarose (Sigma) and then with recombinant Protein A -agarose (Upstate Biotechnology Inc.) and then incubated with 5 μg anti-IR overnight at $4^0$C. Recombinant Protein A-agarose (100 μl of a 50% (v/v) suspension) was added to the mixture and incubated for 2 h at $4^0$C with rotation. Samples were then centrifuged for 1 min at 14 000 x g and the pellet containing anti-insulin receptor-antigen-agarose conjugate was washed three times in a PBS pH 7.3 buffer supplemented with 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.04 μg/ml NaF, and then twice with PBS, 0.04 μg/ml NaF. The washed beads were resuspended in 50 μl of Laemmeli sample buffer (34) with 5% (v/v) 2-mercaptoethanol, boiled for 5 minutes, and centrifuged for 5 minutes at 14 000 x g; the supernatant was then analyzed on 7.5% (w/v) SDS-PAGE followed by western blotting with anti-PY.

### *Phosphosphorylation of nuclear proteins binding to DNA.*

Nuclei extracts were prepared as described above with the exception that phosphatase inhibitors NaF, and $NaP_2O_5$ were removed from all buffers and sodium orthovanadate was lowered from 10 mM and 2 mM to 10 μM in all buffers. The concentration of phosphatase inhibitors found in the former buffers were reduced as the high levels of phosphatase inhibitor was found to produce morphological changes in the isolated nuclei.

DNA-binding proteins were prepared from nuclei extract as described (41). The purified nuclei pellet was resuspended in buffer containing 50 mM Tris-HCl pH 7.4, 250 mM sucrose, 5 mM $MgSO_4$, 1 mM PMSF, 0.0015 TIU/ml aprotinin, and 5 µM leupeptin. DNase 1 and RNase A were added to suspensions to 250 µg/ml final concentration each. After 1 hour of incubation, the nuclei were sedimented at 5 000 x g for 10 min. The supernatant, containing soluble nuclear proteins and weakly DNA-associated proteins, was collected. The pellet was resuspended in a buffer containing 10 mM Tris-HCl pH 7.4, 0.2 mM $MgSO_4$, 1 mM PMSF, and 0.0015 TIU/ml aprotinin. This buffer containing 2.0 M NaCl was then slowly added to a final NaCl concentration of 1.6 M. After 15 min incubation, the residual structures were sedimented at 5000 x g for 20 min. The supernatant, which contained tightly bound DNA proteins, was collected and stored at -70$^0$C. To determine the tyrosine phosphorylation state, 100 µg of DNA binding proteins from each sample was subjected to SDS-PAGE using a 10 % (w/v) polyacrylamide gel. DNA-binding proteins were analyzed by western blotting using anti-phosphotyrosine as described above.

### *Northern Blot Analysis*

Liver taken from 8 wk mice treated as described above was extracted at selected times following oral glucose gavage, immediately frozen in liquid nitrogen and stored at -70$^0$C. Total RNA was extracted from tissue using the guanidium isothiocyanate, phenol/chloroform method (42) using a commercially available TRIZOL reagent (GIBCO BRL, Life Technologies, MD, USA). The quantity and purity of RNA was determined by absorbance at 260 and 280 nm. RNA (30 µg) was analyzed by Northern blotting (43) after electrophoresis on a 1% (w/v) agarose/0.66 M formaldehyde gel and transfer onto a nitrocellulose membrane (Micron Separation Inc., Westborough, MA).

For each blot, equal loading of lanes and integrity of RNA was confirmed by ethidium bromide flourescence of ribosomal 18S and 28S RNA.

The cDNA encoding for malic enzyme was provided by Dr. D.W. Back, (Queen's University, Kingston, Ontario, Canada). The cDNA encoding for fatty acid synthase was provided by Dr. S. Smith, (Children's Hospital Oakland Research Institute, Oakland, California, USA). Probes were labeled with [$\alpha$-$^{32}$P]-dATP using the Random Primer Labeling System (GIBCO BRL) (44). Nitrocellulose filters were hybridized with probe at 65$^0$C and then washed to remove non-specifically bound redioactivity using standard techniques (45). Filters were then exposed to Kodak X-OMAT AR films (Eastman Kodak, Liverpool, UK), for 4-48 hours at 80$^0$C with intensifying screeens.

### *Statistical Evaluation*

For each analysis three experiments were carried out, each on different days. Three different animals were examined for each timepoint selected in each of three experiments ($n = 9$/timepoint). The difference between selected timepoints following oral glucose was assessed using analysis of variance procedures (SAS Version 6.11 SAS Inc., Cary, NC, USA). A significant difference between timepoints was determined by a Duncan's multiple range test (46,47) ($p< 0.05$). There was no significant difference between days when analyzed by analysis of variance procedures.

## *RESULTS*

### ***Animal Characteristics***

Body and liver weights of animals at 8 weeks of age averaged 28.1 ± 0.4 g and 1.18 ± 0.03 g, respectively. Body and liver weights were similar between animals sacrificed after the various periods of time following oral glucose gavage.

### ***Recovery and Purity of Nuclei Preparations***

Recovery of nuclei estimated from DNA determinations was 3.7 ± 0.3 % in nuclei preparations using high concentrations of phosphatase inhibitors and 6.6 ± 0.3 % in nuclei preparations using lower concentrations of phosphatase inhibitors (**Table 4.1**), which are typical values for the percentage recovery of DNA following nuclei isolation from liver (48). Nuclei prepared with buffers containing high amounts of phosphatase inhibitors had 1.6 ± 0 % of plasma membrane 5'nucleotidase activity. Reducing the amount of phosphatase inhibitors in the buffers used for nuclei isolation increased the amount of contamination of nuclei by plasma membrane to 3.8 ± 0.1 % ($p < 0.05$) (**Table 4.2**). The amount of contamination of nuclei by mitochondrial succinate cytochrome c reductase activity was typically less than 1 % (**Table 4.3**). Nuclei prepared from buffers containing high amounts of phosphatase inhibitors had 5.63 ± 0.88 % contamination by endoplasmic reticulum, the nuclei prepared from buffer with lower phosphatase inhibitor concentrations were 7.60 ± 1.26 % contaminated by endoplasmic reticulum (**Table 4.4**). Examination of the DNA-stained nuclear preparations by microscopy revealed that high phosphatase inhibitor concentrations distorted the shape of the nuclei so that the nuclei were no longer round, and caused the nuclei to clump together. These morphological changes were eliminated upon the removal of NaF, and $NaP_2O_5$ and the reduction of $NaVO_3$ from 10 mM and 2 mM, respectively to 10 μM. Isolated nuclear preparations stained by ethidium bromide and methylene blue appeared intact and free of cytoplasmic material when examined by light microscopy. See **Appendix VI** for more details.

### *Insulin and Glucose Determinations*

Fasted animals were given an oral dose of glucose to induce insulin stimulation *in vivo*. Following this dose, serum glucose levels increased from basal levels of 6.1 ± 0.2 mM to 26.9 ± 1.1 mM and 23.5 ± 0.7 mM at 10 and 15 min post gavage respectively ($p<0.05$) (**Figure 4.1**). By 180 minutes after the glucose gavage, glucose levels dropped to 8.9 ± 0.5 mM, but were still significantly greater than basal levels ($p<0.05$). The oral dose of glucose induced a rise of serum insulin from fasting levels 20.3 ± 0.6 pM to 28.7 ± 1.2 pM at 10 min and 29.5 ± 1.0 pM at 15 min following glucose gavage ($p< 0.05$) (**Figure 4.1**). Serum insulin levels remained increased from basal levels at 180 minutes following gavage (27.3 ± 1.8 pM). The increase in both serum glucose and insulin within 10 and 15 minutes following glucose gavage suggests that insulin stimulation of insulin-responsive tissues occurs *in vivo* within that time frame.

### *Nuclear Insulin Receptor Levels*

Anti-insulin receptor antibody recognized insulin receptor in 1 % (v/v) Triton X-100 solublized nuclear extracts. As Triton X-100 at 1 % (v/v) is able to solubilize the outer but not the inner nuclear membrane, this suggests that the insulin receptor is associated with the outer nuclear membrane (23). Western blotting analysis of nuclear extracts at selected timepoints following oral glucose using anti-insulin receptor antibody revealed that levels of insulin receptor increased by 81 % at 15 min following glucose gavage compared to the insulin receptor levels at basal conditions ($p < 0.05$) (**Figure 4.3**). At 180 min, levels of insulin receptor in the nucleus increased 175 % from basal conditions ($p < 0.05$) and were higher than insulin receptor levels found at 15 min ($p < 0.05$).

### *Nuclear Insulin Receptor Autophosphorylation*

Upon insulin binding to the α-subunit, the β-subunit of the insulin receptor undergoes tyrosine phosphorylation. The phosphorylation state of the insulin receptor β-subunit in the hepatocyte nucleus following oral glucose gavage is illustrated (**Figure 4.4**). *In vivo* stimulation increased receptor phosphorylation by 1.5 fold ($p < 0.05$) at 15 minutes and by 2.4 fold at 180 minutes ($p < 0.05$) compared to basal conditions.

### *Nuclear Extract Protein Phosphorylation*

Putative endogenous substrates of the nuclear insulin receptor tyrosine kinase were determined by assessing the *in vivo* tyrosine phosphorylation of DNA-binding proteins in the nucleus, using anti-phosphotyrosine, at selected timepoints following oral glucose gavage. A 31 % decrease in tyrosine phosphorylation of a nuclear protein, ≈ 30 KDa, was observed by 15 min following *in vivo* stimulation (**Figure 4.5**).

### *Insulin Stimulation of Gene Expression*

The effect of *in vivo* insulin stimulation on expression of insulin responsive genes was assessed by determining the levels of mRNA transcripts of these genes detected in mouse liver samples following the oral glucose dose. ME and GAPDH mRNA levels at selected times following glucose gavage were determined by northern blot analysis using cDNA probes for the respective genes. At 15 min following oral glucose gavage ME mRNA was increased by 35 % ($p<0.05$) compared to basal levels, however by 180 min ME mRNA levels were no different from basal levels (**Figure 4.6**). GAPDH mRNA levels increased from basal by 16 % at 15 min ($p<0.05$) and then decreased at 180 min following glucose gavage to those of basal levels (**Figure 4.7**). The level of FAS mRNA however, was not changed from basal at either 15 min or at 180 min following insulin stimulation (data not shown).

## *DISCUSSION*

The present study demonstrates that insulin receptor accumulates in the hepatocyte nucleus following an increase in serum glucose and insulin levels induced by oral glucose administration. An increase in insulin receptor levels and autophosphorylation state was detected in nuclei fractionated to purity at 15 and 180 min following an oral dose of glucose. Within the time course of active insulin receptor accumulation in the cell nucleus was the dephosphorylation of a nuclear DNA-binding phosphoprotein and the transient increase in gene expression of two insulin responsive genes, malic enzyme and glyceraldehyde-3-phosphate dehydrogenase. These results suggest that nuclear insulin receptors may play a role in transmission of the insulin signal to the hepatocyte nucleus.

Insulin receptor levels in the nucleus increased rapidly following *in vivo* insulin stimulation, within 15 min of oral glucose administration. Similar observations have been made in insulin stimulated adipocytes in culture (18). In two earlier studies using cultured hepatocytes (19) and adipocytes (15), the kinetics of insulin receptor internalization rate was slower. Peak accumulation of receptors in the nucleus occurred at 60 min (19) and there appeared to be a time delay of about 30 min of the nuclear translocation of receptor (15,19). The differences in estimated translocation rates may be a function of the different types of probes used to detect trafficking of the insulin receptor in these studies. In the latter studies (15,19), [$^{125-}$I]-labeled insulin was covalently linked to the receptor by photoaffinity labeling prior to internalization of the hormone receptor complex. In the present study and in studies reported in (18), immunodetection methods were applied to detect the insulin receptor after termination of cellular processes by cell fractionation. It may be suggested that covalent linkage of ligand to receptor impaIRE the trafficking of the receptor within the cell. Experiments show that covalently bound insulin causes routing of the insulin-receptor complex to lysosomes and slow and inefficient recycling of the receptor back to the plasma membrane (49,50). The rate of translocation of insulin receptor to the nucleus observed in this study is well within the range of rates reported for the nuclear translocation of other growth factor receptors. In studies where the kinetics of translocation of other cell surface-localized hormone receptors to the nucleus upon ligand stimulation has been reported, the appearance of the relevant receptor in the nucleus varies between 10 to 20 min (23,27,28) and 1 to 2 hr (24,25).

Despite accumulating evidence, the idea that insulin receptors are translocated to the nucleus during the process of ligand-stimulated internalization is controversial (18,52). It is possible that receptors are detected at the nucleus via contamination from plasma membrane during fractionation process involved in nuclear isolation. However many investigators report nuclei preparations that are free of plasma membrane contamination (18,20). In the present study the recovery of plasma membrane 5'nucleotidase activity in nuclear fractions appears consistently to be less than 4 % (i.e. the specific activity measured in the nuclei divided by the specific activity in isolated plasma membrane), and is not increased by the oral glucose gavage. This minimal contamination could not account for the increase in insulin receptor observed in the nucleus following *in vivo* stimulation after oral glucose gavage. Using the same criteria, contamination by mitochondria was estimated to be less than one percent, however there was significant contamination by microsomal membrane (10 %), as determined by glucose-6-phosphatase. This finding is to be expected as the membranes of the endoplasmic reticulum are continuous with the outer nuclear membrane (53).

Increase of the insulin receptor content of the hepatocyte nucleus following oral glucose gavage was associated with an increase in the β-subunit tyrosine phosphorylation during the same time period. An increase in nuclear insulin receptor β-subunit phosphorylation upon insulin stimulation has been reported earlier (18,20) and this is demonstated in the present study. In one study using adipocytes in culture (18), 1 nM insulin stimulated this process two fold by 5 min. These findings are consistant with the observations made *in* *vivo* in the present study (1.5 fold within 15 min). In comparison, a four fold increase in insulin receptor phosphorylation in plasma membrane of rat liver was observed following insulin administration to intact animals (14). In the study of Kim et al. (18), and in the present study, the time course of insulin receptor nuclear accumulation and the time course of increase in appearance of a tyrosine phosphorylated β-subunit in the nucleus are similar implying that the receptor arrives in the nuclear membrane in a phosphorylated state. In association with the period of appearance of phosphorylated insulin receptor β-subunit in the hepatocyte nucleus, a decrease in tyrosine phosphorylation of a nuclear DNA-binding protein of about 30 KDa was detected by immunoblotting with anti-phosphotyrosine. Dephosphorylation of a 30 KDa phosphotyrosine nuclear protein upon insulin stimulation has not been reported. Insulin stimulates the phosphorylation (54-58) and dephosphorylation (59,60) of several nuclear proteins and transcription factors. In one study (59), addition of insulin to the nuclear extracts induced a 43 % decrease in the phosphorylation of nuclear proteins, comparable to the observations (31 % decrease) made in the present study. Insulin-induced changes in phosphorylation of these proteins occurs on serine and threonine residues (54-59), suggesting that these phosphoproteins are not direct substrates of the insulin receptor tyrosine kinase. Modification of nuclear proteins by tyrosine phosphorylation is a regulatory mechanism used by other hormones including insulin-like growth factor-1 (60), growth hormone (61), epidermal growth factor (62). Insulin-induced tyrosine phosphate modification of a nuclear protein (82 KDa) which is related to c-Fos has been reported (57), however such a protein was not detected in the present study. The changes in phospho-tyrosine content of a nuclear protein in the present study within the time frame of the appearance of insulin receptor tyrosine kinase in the nucleus suggests that these changes may have been catalyzed by a tyrosine phosphatase which is directly regulated by the insulin receptor on the nuclear membrane.

In the present study an increase of ME and GAPDH mRNA, but not of FAS mRNA was observed within 15 min after the oral glucose meal. The increase in gene expression observed in this study occurred in parallel to the changes associated with insulin receptor translocation to the nucleus. Although insulin receptor levels in the nucleus continued to increase at 180 min following the glucose meal, the increase in mRNA levels appeared to be transient. It must be noted that tyrosine phosphorylation of the β-subunit was not increased at 180 min when compared to the tyrosine phosphorylation at 15 min following the glucose meal. This finding suggests that the induction of mRNA synthesis may be correlated to the tyrosine phosphorylation state of insulin receptor in the nucleus. Measurement of the time course of insulin induction of insulin responsive genes *in vivo* (63-65), and in cultured cells (66-68) has been reported. In most of these studies, detection of changes in gene expression begins between 2 - 4 hr following stimulation (63-65,67,68). In contrast, insulin was demonstrated to increase c-fos gene expression within 5 min in cultured hepatoma cells (66). The differences in time required for induction of insulin-responsive gene may reflect differences in mechanisms involved in insulin signaling to nucleus. For insulin-responsive gene expression to be affected swiftly following insulin stimulation, the mechanisms involved must be rapid such as those mediated by phosphorylation/dephosphorylation cascades and not those pathways requiring de novo protein synthesis (5). There may be also be specific nutrient-hormonal interactions required for the insulin response. For some genes to be regulated by insulin the presence of glucose is required (5). Athough the changes observed in the present study in malic enzyme gene expression were modest (35 % increase from basal), they were comparable to those reported in a similar study. Administration of insulin by injection caused an increase in ME gene expression of approximately 100% (70).

The present study used a model of *in vivo* stimulation of insulin receptors, via an oral glucose dose, to show that insulin receptor signaling to the nucleus involves nuclear translocation of the activated insulin receptor tyrosine kinase. This study is the firstt to demonstrate ligand-dependent nuclear translocation of the insulin receptor *in vivo*, concomitant with *in vivo* insulin-induced nuclear responses. The implications of this study are that rapid signaling to the nucleus exists that may be mediated by nuclear translocation of the insulin receptor tyrosine kinase. Direct signaling to the nuclear membrane via nuclear translocation of the insulin receptor offers a novel pathway by which dietary carbohydrate intake has rapid effects on gene expression in the liver.

## *REFERENCES*

1. Czech, M.P. (1985) Annu. Rev. Physiol., 47:357-381.

2. Kasuga, M., Hedo, J.A., Yamada, K.M., and Kahn, C.R. (1982) Science, 215:185-186.

3. Cheatham, B., and Khan, C.R. Insulin action and the insulin signaling network. (1995) Endocr. Rev., 16:117-142.

4. White, M. F., and Kahn, C.R. The insulin signaling system. (1994) J. Biol. Chem., 269:1-4.

5. O'Brien, R.M., and Granner, D.K. Regulation of gene expression by insulin. (1996) Physiol. Rev.,76:1109-1161.

6. Denton, R.M. and Tavare, J.M. Does mitogen-activated-protein kinase have a role in insulin action? The cases for and against. (1995) Eur. J. Biochem., 227:597-611.

7. Sun, X. J. Rothenberg, P.L., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J., and White, M. (1991) Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature, 352:73-77.

8. Araki, E.M., Lipes, M.A., Patti, M.-E., Bruning, J.C., Haag, B.III, Johnson, R.S., and Kahn, C.R. (1994) Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature Lond., 372:186-190.

9. Tamemoto, H., Kadowaki, H.T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi., Y., Satoh, S., Sekihara, H., Yoshioka, S., Horikoshi, H., Furuta, Y., I-Kawa, Y., Kasuga, M., Yazaki, Y., and Aizawa, S. (1994) Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature Lond., 372:182-186.

10. Sutherland, C., O'Brien, R.M., and Granner, D.K. (1995) Phosphatidylinositol 3-kinase, but not p70/p85 ribosomal S6 protein kinase, is required for the regulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression by insulin. Dissociation of signalling pathways for insulin and phorbol ester regulation of PEPCK gene expression. J. Biol. Chem., 270(26):15501-15506.

11. Yang, S.H., and Dickson, A.J. Inhibitors of signalling identify differential control processes responsible for selective effects of insulin on the expression of phosphoenolpyruvate carboxykinase and gene 33 in rat H4 hepatoma cells. (1995) Biochem. J., 310(2):375-378.

12. Knutson, V.P. Cellular trafficking and processing of the insulin receptor. (1991) FASEB J., 5:2130-2138.

13. Backer, J.M., Sheolson, S.E., Haring, E., and White, M.F. Insulin receptors internalize by a rapid, saturable pathway requiring receptor autophosphorylation and an intact juxtamembrane region. (1991) J. Cell. Biol., 115(6):1535-1545.

14. Khan, M.N., Baquiran, G., Brule, C., Burgess, J., Foster, B., Bergeron, J.J.M., and Posner, B.I. Internalization and activation of the rat liver insulin receptor kinase *in vivo*. (1989) J. Biol. Chem., 264(22):12931-12940.

15. Smith, R.M., and Jarett, L. Ultrastructural evidence for the accumulation of insulin in nuclei of intact 3t3-L1 adipocytes by an insulin-receptor mediated process. (1987) Proc., Natl., Acad., Sci., U.S.A., 84:459-463.

16. Horvat, A. Insulin binding sites on rat liver nuclear membranes: Biochemical and immunoflourescent studies. (1978) J. Cell. Physiol., 97:37-48.

17. Vigneri, R., Goldfine, I.D., Wong, K.Y., Smith, G.J., and Pezzino, V. The Nuclear Envelope. The major site of insulin binding in rat liver nuclei. (1978) J. Biol. Chem., 253(7);2098-2103.

18. Kim, S.-J., and Kahn, C.R. Insulin induces rapid accumulation of insulin receptors and increases tyrosine kinase activity in the nucleus of cultures adipocytes. (1993) J. Cell. Physiol., 157:217-228.

19. Podlecki, D.A., Smith, R.M., Kao, M., Tsai, P., Huecksteadt, T., Brandenburg, D., Sasher, R.S., Jarett, L., and Olefsky, J.M. Nuclear translocation of the insulin receptor. A possible mediator of insulin's long term effects. (1987) J. Biol. Chem., 292:3362-3363.

20. Wong, K.Y., Hawley, D., Vigneri, R., Goldfine, I.D. Comparison of solubilization and purified plasma membrane and nuclear insulin receptors. (1988) Biochemistry, 27:375-379.

21. Smith, R.M., and Jarett, L. Receptor-mediated endocytosis and intracellular processing of insulin: ultrastructural and biochemical evidence for cell-specific heterogeneity and distinction from non-hormonal ligands. (1988) Lab. Invest. 58:613-629.

22. Cheema, S.K., Venkatraman, J.T., and Clandinin, M.T. Insulin binding to liver nuclei from lean and obese mice is altered by dietary fat. (1992) B.B.A., 1117:37-41.

23. Lobie, P.E., Wood, T.J.J., Chen, C.M., Waters, M.J., and Norstedt, G. Nuclear translocation and anchorage of the growth hormone receptor. (1994) J. Biol. Chem.,269(50):31735-31746.

24. Jiang, L-W., and Schindler, M. Nucleocytoplasmic transport is enhanced concomitant with nuclear accumulation of epidermal growth factor (EGF) binding activity in both 3T3-L1 and EGF receptor reconstituted NR-6 fibroblasts. (1990) J. Cell Biol.,110:559-568.

25. Rakowicz-Szulczynska, E.M., Rodeck, U., Herlyn, M., and Koprowski, H. Chromatin binding of epidermal growth factor, nerve growth factor, and platelet-derived growth factor in cells bearing the appropriate surface receptors. (1986) Proc. Natl. Acad. Sci. USA,83:3728-3732.

26. Buckley, A.R., Montogomery, D.W., Hendrix, M.J.C., Zukoski, C.F., and Putnam, C.W. Identification of prolactin receptors in hepatic nuclei. (1992) Arch. Biochem. Biophys.,296(1):198-206.

27. Bouche, G., Gas, N., Prats, H., Baldin, V., Tauber, J-P., Teissie, J., and Amalric, F. Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing $G_0 \rightarrow G_1$ transition. (1987) Proc. Natl. Acad. Sci. USA,84:6770-6774.

28. Yanker, B.A., and Shooter, E.M. Nerve growth factor in the nucleus: Interaction with receptors in the nuclear membrane. (1979) Proc. Natl. Acad. Sci. USA,76(3):1269-1273.

29. Cheema, S.K., and Clandinin, M.T. Diet and diabetes induced change in insulin binding to the nuclear membrane in spontaneously diabetic rats in association with change in fatty acid composition of phosphatidylinositol. Biochim., Biophys., Acta. Submitted.

30. Helmerhorst, E., and Nakhoul, A.-M. Novel affinity support prepared by crosslinking purified insulin receptors to agarose. (1993) Biochem. Biophys. Res. Comm., 195(1):237-241.

31. Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y., and Itakura, K. Purification of insulin receptor with full binding activity. (1983) J. Biol. Chem., 258(8):5045-5049

32. Desbuquois, B., and Aurbach, G.D. Use of polyethylene glyeol to separate free and antibody bound peptide hormones in radioimmunoassays. (1971) J. Clin. Endocr., 33:732-738.

33. Assil, H.I., Sheth, H., and Sporns, P. An ELISA for sulfonamide detectioin using affinity-purified polyclonal antibodies. (1992) Food Res. Int., 25:343-353.

34. Blobel, G., and Potter, V.R. Nuclei from rat liver: isolation method that combines purity wth high yield. (1966) Science, 154:1662-1665.

35. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the Folin phenol reagent. (1951) J. Biol. Chem., 193:265-275.

36. Aronson, N.N.Jr., and Touster, O. Isolation of rat liver plasma membrane fragments in isotonic sucrose. (1974) Methods in Enzymology, 31:90-102.

37. Green, D.E., Mii, S., and Kohout, P.M. Studies on the terminal electron transport system. I) Succinate dehydrogenase (1955) J., Biol., Chem., 551-567.

38. Swanson, M.A. (1955) Meth. Enzymol.,2:541-543.

39. Burton, B.J.(1956) Biochem. J., 62:315-318.

40. Laemmli, U.K. (1970) Nature (London),227:680-685.

41. Jaumot, M., Agell, N., and Bachs, O. Putative nuclear cdk2 substates in normal and transformed cells. Biochem., Biophys., Res., Comm., (1996) 219(6):560-564.

42. Chromczynski, P., and Sacchi, N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. (1987) Anal. Biochem., 162:156-159.

43. Seed, B. Attachment of nucleic acids to nitrocellulose and diazonium substituted supports. In Genetic engineering: Principles and methods. (1979)(ed. J.K. Setlow and A. Hollaender), vol. 4, p 91-102. Plenum Publishing, New York. NY, USA.

44. Feinberg, A.P., and Vogelstein, B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. 1983, Anal. Biochem., 132:6-13.

45. Wreshner, D.H., and Herzberg, M. A new blotting medium for the simple isolation and identification of highly resolved messenger RNA. (1984) Nucleic Acids Res., 12:1349-59.

46. Duncan, D.B. Multiple range and multiple *F* tests. (1955) Biom.,11:1-42

47. Steel, R.G.D., and Torrie, J.H. (1980) in Principles and Procedures of Statistics, 2nd edn., McGraw-Hill Book Co., Ch. 8-9, New York, NY, USA.

48. Graham, J.M. The isolation of nuclei and nuclear membranes from rat liver. In: Methods in Molecular Biology, Vol. 19: Biomembrane Protocols: I. Isolation and Analysis. J.M.Graham, and J.A. Higgins eds. (1993) Humana Press Inc., Totowa, N.J. pp, 19-28.

49. Fehlmann, M., Carpentier, J.-L., Van Obberghen, E., et al. Internalized insulin receptor are recycled to the cell surface in rat hepatocytes. (1982) Proc. Natl. Acad Sci. U.S.A.,79:5921-5925.

50. Carpentier, J.-L., Gazzano, H., Van Obberghen, E., Fehlmann, M., Freychet, P., Orci, L. Intracellular pathway by the insulin receptor covalently coupled to $^{125}$I-photoreactive insulin during internalization and recycling. (1986) J. Cell. Biol.,102:989-996.

51. Franke, W.W., Scheer, U., Krohne, G., and Jarasch, E. The nuclear envelope and the architecture of the nuclear periphery. (1981) J. Cell. Biol., 91:39s.

52. Soler, A.P., Thompson, K.A., Smith, R.M., and Jarett, L. Immunological demonstration of the accumulation of insulin, but not insulin receptors, in nuclei of insulin-treated cells. (1989) Proc. Natl. Acad. Sci U.S.A.,86:6640-6644.

53. Reush, J.E., Hsieh, P., Klemm, D., Heoffler, J., and Draznin, B. Insulin inhibits dephosphorylation of 3',5'-monophosphate response element binding protein/activating transcription factor-1: effect on nuclear prosphoserine phosphatase 2a. (1994) Endocrinol.,135(6):2418-22.

54. Reucsh, J.E.B., Hsieh, P., Bhuripanyo, P., Carel, K., Leitner, J.W., Olefsky, J.M., and Draznin, B. Insulin inhibits nuclear phosphatase activity: Requirement for the C-terminal domain of the insulin receptor. (1995) Endocrinol., 136:2464-2469.

55. Csermely, P., Schnaider, T., Cheatham, B., Olson, M.O.J., and Kahn, C.R. Insulin induces the phosphorylation of nucleolin. A possible mechanism of insulin induced RNA-efflux from nuclei from nuclei. (1993) J. Biol. Chem., 268(13):9747-9752.

56. Kim, S.J., and Kahn, C.R. Insulin stimulates phosphorylation of c-Jun, c-Fos, and Fos-related proteins in cultured adipocytes. (1994) J. Biol. Chem., 269(16):11887-11892.

57. Csermely, P., and Kahn, C.R. Insulin induces the phosphorylation of DNA-binding nuclear proteins including lamins in 3T3-F442A. (1992) Biochem.,31:9940-9946.

58. Daniel, S., Zhang, S., DePaoli-Roach, A.A., and Kim, K-H. Dephosphorylation of Sp1 by protein phosphatase 1 is involved in the glucose-mediated activation of the acetyl-coA carboxylase gene. (1996) J. Biol. Chem., 271(25):14692-14697.

59. Purrello, F., Burnham, D.B., and Goldfine, I.D. Insulin regulation of protein phosphorylation in isolated rat liver nuclear envelopes: Potential relationship to mRNA metabolism. (1983) Proc. Natl. Acad. Sci. USA, 80:1189-1193.

60. Takahashi, K., and Suzuki, K. Association of insulin-like growth factor-1-induced DNA-synthesis with phosphorylation and nuclear extrusion of p53 in human breast cancer MCF-7 cell. (1993) Int. J. Cancer,55(3):453-458

61. Waxman, D.J., Ram, P.A., Park, S.H., and Choi, H.K. Intermittent plasma growth hormone triggers tyrosine phosphorylation and nuclear translocation of a liver-expressed, Stat 5-related DNA binding protein. Proposed role as an intracellular regulator of male-specific liver gene transcription. (1995) J. Biol. Chem.,270(22):13262-13270.

62. Ruff-Jamison, S., Chen, K., and Cohen, S. Epidermal growth factor induces the tyrosine phosphorylation and nuclear translocation of Stat 5 in mouse liver. (1995) Proc. Natl. Acad. Sci. USA,92(10):4215-4218.

63. Saladin, R., De Vos, P., Guerre-Millo, M., Leturque, A., Girard, J., Staels, B., and Auwerx, J. Transient increase in obese gene expression after food intake or insulin administration. (1995) Nature,377:527-529.

64. MacDougald, O.A., Hwang, C-S., Fan, H., and Lane, M.D. Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. (1995) Proc. Natl. Acad. Sci. USA,92:9034-9037.

65. Iritani, N., Fukuka, H., Tada, K., Itoh, A., and Noguchi, T. Diet differentially regulates glucokinase and L-type pyruvate kinase gene expression in rat liver. (1995) J. Nutr.,125:2945-2952.

66. Messina, J.L. Insulin's regulation of *c-fos* gene transcription in hepatoma cells. (1990) J. Biol. Chem.,265(20):11700-11705.

67. Lin, M.C.M., Gordon, D., and Wetterau, J.R. Microsomal triglyceride transfer protein (MTP) regulation in HepG2 cells: insulin negatively regulates MTP gene expression. (1995) J. Lipid. Res.,36:1073-1081.

68. Stanley, F.M. An element in the prolactin promoter mediates the stimulatory effect of insulin in transcription of the prolactin gene. (1992) J. Biol. Chem.,267(23):16719-16726.

69. Garcia-Jimenez, C., Benito, B., Jolin, T., and Santisteban, P. Insulin regulation of malic enzyme gene expression in rat liver: Evidence for nuclear proteins that bind to two putative insulin response elements. (1994) Mol. Endocrinol.,8:1361-1369.

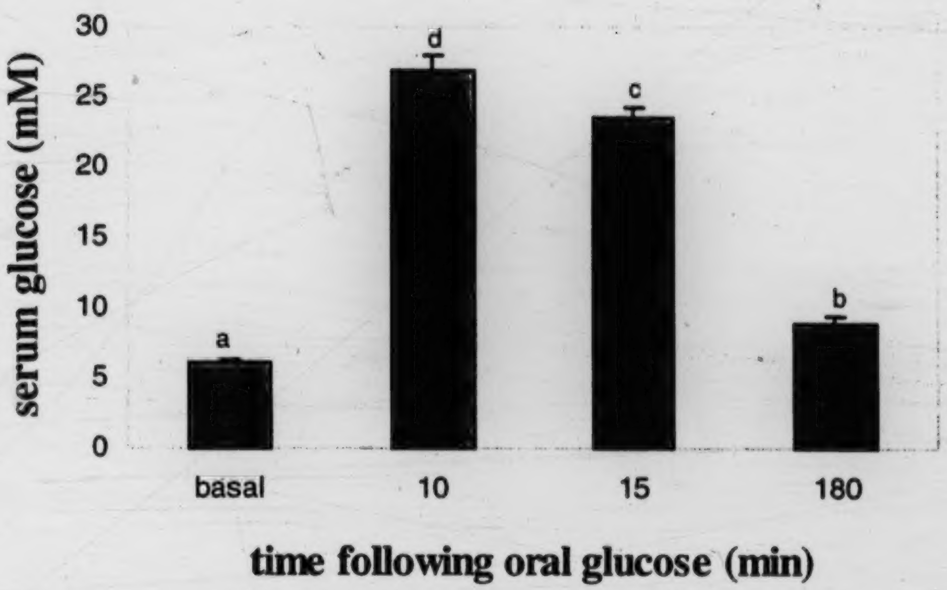


**Figure 4.1 Mouse serum glucose measurements at selected times following oral glucose gavage.** Eight week old mice were given an oral glucose gavage of 3 g/kg body weight. At 0, 10, 15, and 180 min following gavage, mice were anestitized and blood removed by cardiac puncture. Serum glucose is expressed in mM. Data represents means ± standard error. Values with different letters are significantly different ($p<0.05$), n=9 for each timepoint.

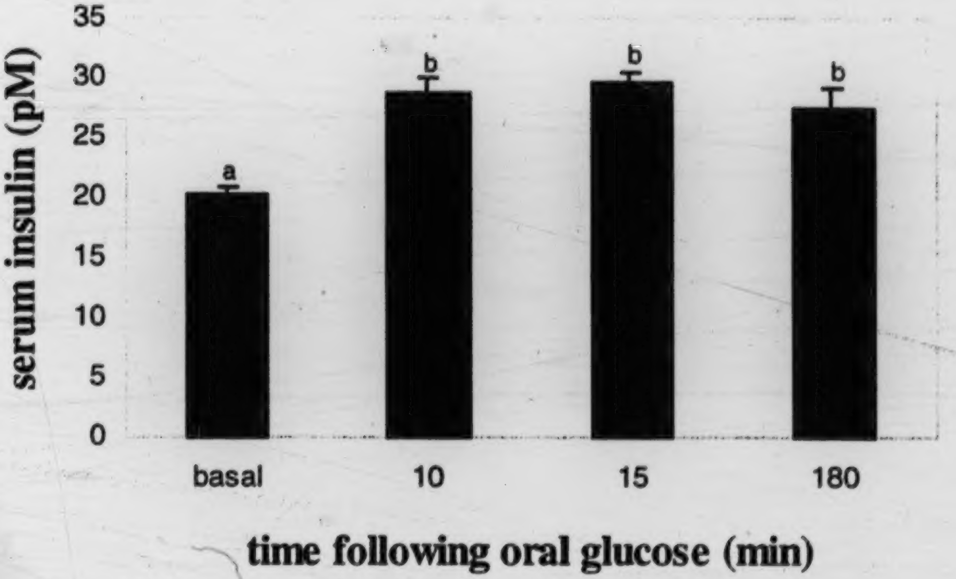


**Figure 4.2 Mouse serum insulin measurements at selected times following oral glucose gavage.** Eight week old mice were given an oral glucose gavage of 3 g/kg body weight. At 0, 10, 15, and 180 min following gavage, mice were anesthetized and blood removed by cardiac puncture. Serum insulin concentrations are expressed in pM. Data represents means ± standard error. Values with different letters are significantly different ($p<0.05$), n=9 for each timepoint.

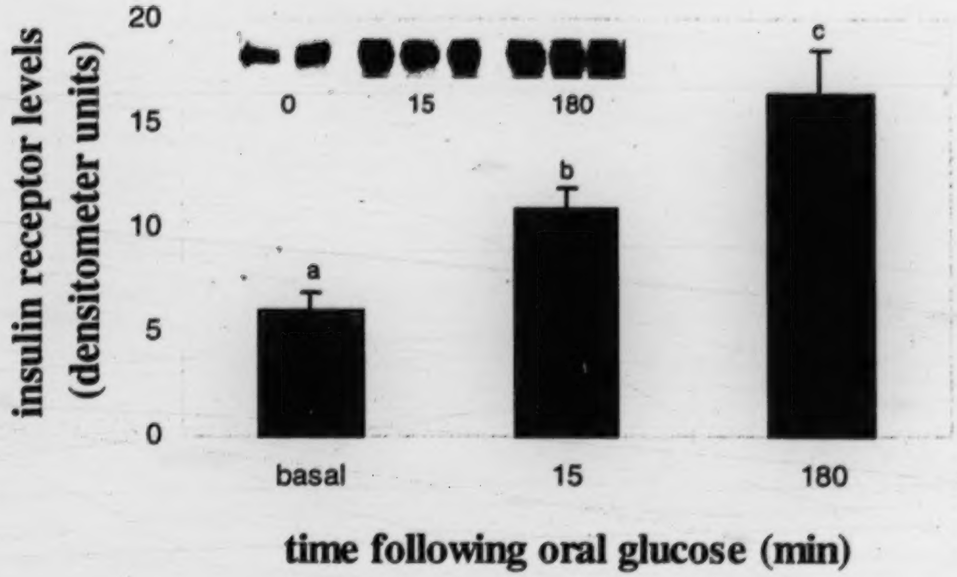


**Figure 4.3 Insulin receptor levels in the hepatocyte nucleus at selected times following oral glucose gavage.** Eight week old mice were given an oral glucose gavage of 3 g/kg body weight. At 0, 10, 15, and 180 min following gavage, mice were sacrificed and liver nuclei isolated. Insulin receptor levels were determined by western blotting using anti-insulin receptor antibody. Values are expressed in densitometer units. Data represents means ± standard error. Values with different letters are significantly different ($p<0.05$), n=9 for each timepoint.

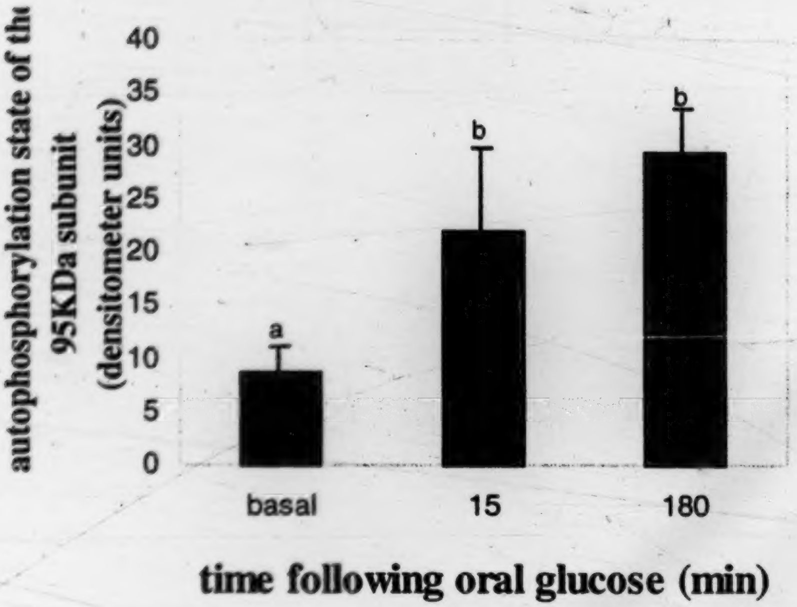


**Figure 4.4 Autophosphorylation of the insulin receptor 95K subunit in the hepatocyte nucleus at selected times following oral glucose gavage.** Eight week old mice were given an oral glucose gavage of 3 g/kg body weight. At 0, 15 and 180 min following gavage, mice were sacrificed and liver nuclei isolated. Tyrosine phosphorylation of the 95K subunit of the insulin receptor was determined by immunoprecipitation of nuclei extracts using anti-insulin receptor antibody and then western blotting using anti-phophotyrosine. Data represents means ± standard error. Values are expressed in densitometer units. Values with different letters are significantly different ($p<0.05$), $n=9$ for each timepoint.

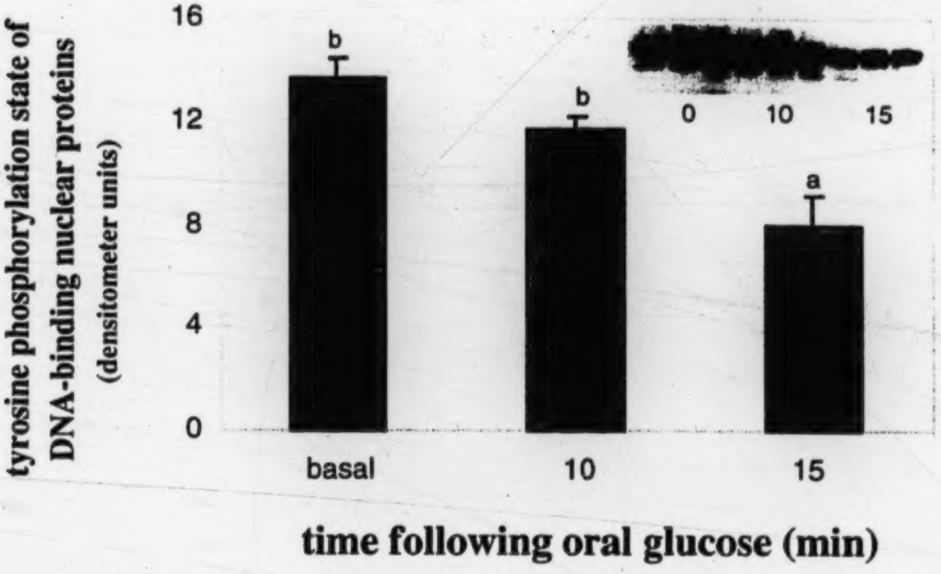


**Figure 4.5 Tyrosine phosphorylation of DNA-binding proteins in the hepatocyte nucleus at selected times following oral glucose gavage.** Eight week old mice were given an oral glucose gavage of 3 g/kg body weight. At 0, 10 and 15 min following gavage, mice were sacrificed and liver nuclei isolated. DNA-binding proteins were prepared and tyrosine phosphorylation of these determined by western blotting with anti-phosphotyrosine. Values are expressed in densitometer units. Data represents means ± standard error. Values with different letters are significantly different ($p<0.05$), $n=9$ for each timepoint.

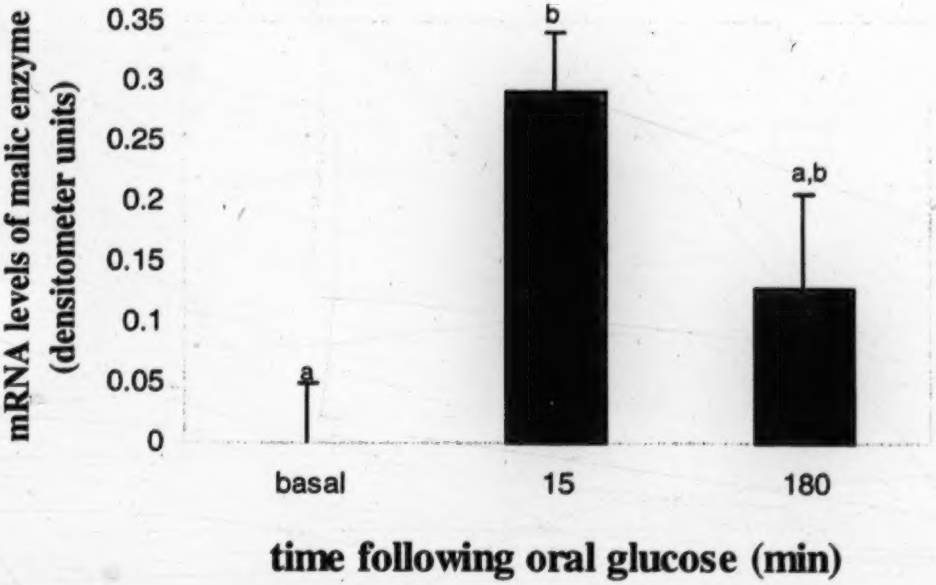


**Figure 4.6 Gene expession of malic enzyme at selected times following oral glucose gavage.** Eight week old mice were given an oral glucose gavage of 3 g/kg body weight. At 0, 15 and 180 min following gavage, mice were sacrificed and liver extracted. mRNA levels of malic enzyme were determined by northern blotting. Values are expressed as a change from basal in densitometer units (0 min = 0.836). Data represents means ± standard error. Values with different letters are significantly different ($p<0.05$), n=9 for each timepoint.

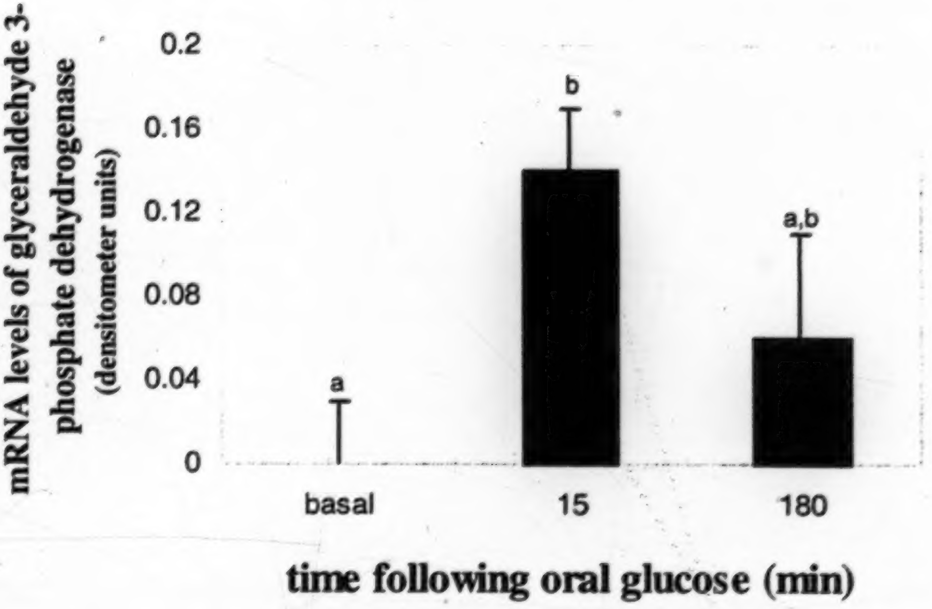


**Figure 4.7 Gene expession of glyceraldehyde 3-phosphate dehydrogenase at selected times following oral glucose gavage.** Eight week old mice were given an oral glucose gavage of 3 g/kg body weight. At 0, 15 and 180 min following gavage, mice were sacrificed and liver extracted. mRNA levels of malic enzyme were determined by northern blotting. Values are expressed as a change from basal in densitometer units (0 min = 0.909). Data represents means ± standard error. Values with different letters are significantly different ($p<0.05$), n=9 for each timepoint.

**Table 4.1 Effect of Phosphatase Inhibitors on Yield of Nuclei Isolated from Mouse Liver**

| | Total DNA (mg per liver) | Yield |
|---|---|---|
| Homogenate | | |
| No PI | 16.9 ± 1.0 | 100 |
| Low PI | 13.8 ± 0.5 | 100 |
| High PI | 12.7 ± 1.4 | 100 |
| Nuclei | | |
| No PI | 1.4 ± 0.1 | 8.3 ± 0.5 |
| Low PI | 0.90 ± 0.0 | 6.6 ± 0.3* |
| High PI | 0.46 ± 0.0 | 3.7 ± 0.3 |

Values are mean ± standard error of the mean (n=4). Nuclei extracts were isolated from mouse liver in buffers containing either phosphatase inhibitors, NaF, $NaP_2O_5$, and sodium orthovanadate (High PI) or containing sodium orthovanadate only (Low PI). Yield of nuclei was determined by dividing total DNA in nuclei fraction by total DNA in crude homogenate and multiplying by 100. Significant differences were determined using Student's *t*-test ($p < 0.05$). (*) indicates that there was a significant difference between yield obtained from isolation of nuclei in low phosphatase inhibitor containing buffer versus high phosphatase inhibitor containing buffer.

**TABLE 4.2 Effect of Phosphatase Buffers on Purification of Nuclei from Mouse Liver: Contamination by Plasma Membrane**

| | Total Protein (mg) | Total Activity (μmol/min) | Specific Activity (μmol/min/mg) | **Yield** (%) | Fold Purification |
|---|---|---|---|---|---|
| **Homogenate** | | | | | |
| Low PI | 185.0 ± 2.9 | 5.97 ± 0.09 | 0.032 ± 0.001 | 100 | 1 |
| High PI | 174.6 ± 1.3 | 19.9± 0.18 | 0.114 ± 0.000 | 100 | 1 |
| **Plasma Membrane** | | | | | |
| Low PI | 0.54 ± 0.01 | 0.53 ± 0.01 | 0.977 ± 0.009 | 8.89 ± 0.27 | 30.3 ± 0.7 |
| High PI | 0.51 ± 0.02 | 0.50 ± 0.01 | 0.996 ± 0.008 | 2.53 ± 0.06 | 8.74 ± 0.07 |
| **Nuclei** | | | | | |
| Low PI | 1.58 ± 0.09 | 0.06 ± 0.0 | 0.037 ± 0.001 | 0.98 ± 0.04 | 1.15 ± 0.01 |
| High PI | 1.02 ± 0.12 | 0.02 ± 0.0 | 0.016 ± 0.001 | 0.08 ± 0.21 | 0.14 ± 0.00 |

Values are mean ± standard error of the mean (n=4). Nuclei extracts were isolated from mouse liver in buffers containing either phosphatase inhibitors, NaF, $NaP_2O_5$, and sodium orthovanadate (High PI) or containing sodium orthovanadate only (Low PI). Contamination of isolated nuclei by plasma membrane was determined using 5'nucleotidase assay. Specific activity was calculated as the ratio of total enzyme units to total protein. Yield was calculated as the ratio between the total activity of the fraction of interest to the total activity in the crude homogenate and multiplied by 100. Fold purification was calculated as the specific activity of the fraction of interest divided by the specific activity of the homogenate. Relative specific activity was calculated as the specific activity of the nuclei fraction divided by the specific activity of the plasma membrane. Significant differences were determined using Student's *t*-test ($p < 0.05$). (*) indicates that there was a significant difference between relative specific activities of nuclei isolated in low phosphatase inhibitor containing buffer versus in high phosphatase inhibitor containing buffer. N.D = not determined.

**Table 4.3 Effect of Phosphatase Inhibitors on Purification of Nuclei from Mouse Liver: Contamination by Mitochondria**

| | Total Protein (mg) | Total Activity (nmol/min) | Specific Activity (nmol/min/mg protein) | **Yield** (%) | Fold Purification |
|---|---|---|---|---|---|
| **Homogenate** | | | | | |
| **Low PI** | 185.0 ± 2.9 | 19015 ± 3642 | 103.4 ± 20.6 | 100 | 1 |
| **High PI** | 174.6 ± 1.3 | 12448 ± 1973 | 71.4 ± 11.4 | 100 | 1 |
| **Nuclei** | | | | | |
| **Low PI** | 1.58 ± 0.09 | 26 ± 6 | 17.0 ± 5.2 | 0.13 ± 0.01 | 0.15 ± 0.02 |
| **High PI** | 1.02 ± 0.12 | 23 ± 1 | 23.6 ± 4.4 | 0.19 ± 0.03 | 0.35 ± 0.07 |

Values are mean ± standard error of the mean (n=4). Nuclei extracts were isolated from mouse liver in buffers containing either phosphatase inhibitors, NaF, $NaP_2O_5$, and sodium orthovanadate (High PI) or containing sodium orthovanadate only (Low PI). Contamination of isolated nuclei by mitochondria was determined using succinate cytochrome c reductase assay. Specific activity was calculated as the ratio of total enzyme units to total protein. Yield was calculated as the ratio between the total activity of the fraction of interest to the total activity in the crude homogenate and multiplied by 100. Fold purification was calculated as the specific activity of the fraction of interest divided by the specific activity of the homogenate. Significant differences were determined using Student's *t*-test ($p < 0.05$).

**Table 4.4 Effect of Phosphatase Inhibitors on Purification of Nuclei from Mouse Liver: Contamination by Endoplasmic Reticulum**

| | Total Protein (mg) | Total Activity (µmol/min) | Specific Activity (µmol/min/mg protein) | **Yield** (%) | Fold Purification |
|---|---|---|---|---|---|
| **Homogenate** | | | | | |
| **Low PI** | 185.0 ± 2.9 | 204.5 ± 7.2 | 1.10 ± 0.02 | 100 | 1 |
| **High PI** | 174.6 ± 1.3 | 159.6 ± 6.8 | 0.91 ± 0.04 | 100 | 1 |
| **Nuclei** | | | | | |
| **Low PI** | 1.58 ± 0.09 | 15.3 ± 2.1 | 9.98 ± 1.91 | 7.60 ± 1.26 | 9.15 ± 1.90 |
| **High PI** | 1.02 ± 0.12 | 8.8 ± 1.1 | 8.73 ± 0.61 | 5.63 ± 0.88 | 9.68 ± 1.14 |

Values are mean ± standard error of the mean (n=4). Nuclei extracts were isolated from mouse liver in buffers containing either phosphatase inhibitors, NaF, $NaP_2O_5$, and sodium orthovanadate (High PI) or containing sodium orthovanadate only (Low PI). Contamination of isolated nuclei by endoplasmic reticulum was determined using glucose 6-phosphatase assay. Specific activity was calculated as the ratio of total enzyme units to total protein. Yield was calculated as the ratio between the total activity of the fraction of interest to the total activity in the crude homogenate and multiplied by 100. Fold purification was calculated as the specific activity of the fraction of interest divided by the specific activity of the homogenate. Significant differences were determined using Student's *t*-test ($p < 0.05$).

# CHAPTER V

# INSULIN RECEPTOR INCUBATION WITH ISOLATED NUCLEI INDUCES A DECREASE IN NUCLEAR PROTEIN PHOSPHORYLATION AND AN INCREASE IN TRANSCRIPTION FACTOR ASSOCIATION TO DNA.

## *INTRODUCTION*

Regulation of transcription by insulin is achieved by modulation of the binding affinity of transcription factors to specific sequences within the promotor regions of insulin responsive genes (1). These promotor sequences, called insulin responsive elements (IRE), have been identified for genes including glyceraldehyde 3-phosphate dehydrogenase GADPH (2), fatty acid synthase FAS (3), phosphoenolpyruvate carboxykinase PEPCK (4), and malic enzyme ME (5). IREs present in GAPDH (located between -461 to -453) and ME (located between -161 to -170) genes share some homology and may be recognized by similar DNA-binding proteins (5).

Transcription factor function is regulated by post-translational modification including changes in phosphorylation (6). Insulin treatment of the cell alters the phosphorylation state of nuclear proteins such as lamins (7), nucleolin (8), and activator protein-1 (AP-1) transcription factors (9). Regulation of these proteins may be mediated by various protein kinases or phosphatases which become activated upon insulin stimulation of the cell.

Insulin binding and insulin receptor tyrosine kinase activation occurs at the cell surface (10). Insulin signaling within the cell is subsequent to insulin receptor tyrosine kinase activation of its most immediate substrate, insulin receptor substrate (IRS-1) (11). The activity of IRS-1 is thought to be responsible for the transmission of insulin signal to other mediators, including phosphatidylinositol 3'-kinase (P-I-3'K), and mitogen activated protein kinase (MAP-K), within the cell (12). Gene regulation by insulin is maintained in animals deficient in IRS-1 (13,14), which suggests that the existence of other signaling pathways that bypass IRS-1. Upon insulin stimulation of the cell, two distinct intracellular pathways are activated via IRS-1. Members of the mitogen-activated protein kinase (MAP-K) family are central to one pathway, and seem to be implicated in activation of c-fos (15) and prolactin (16) gene expression. For other genes such as PEPCK (17) and hexokinase (18), inactivation of the MAP-K pathway does not abolish the effect of insulin on gene expression. Phosphatidylinositol 3'-kinase (P-I-3'K) is a key enzyme in another insulin-stimulated signaling pathway that has been implicated to be responsible for insulin regulation of PEPCK (19) and hexokinase gene expression (18). In one report (20) however, inactivation of P-I-3'K failed to ameliorate insulin regulation of PEPCK gene expression. Also, p70/p85 ribosomal S6 protein kinase (p $70^{S6K}$), an enzyme thought to lie downstream of P-I-3'K, was inactivated without any effect on insulin regulation of PEPCK gene expression (19). Thus the roles of IRS-1, MAP-K and P-I-3'K activation in the insulin gene regulation are not clear and a pathway from the cell surface to the nucleus resulting in the activation of a specific transcription factor has not been described (1).

There is accumulating evidence that insulin (21-23), and other polypeptide hormones (24-26), activate nuclear processes by direct translocation of activated receptors to the nucleus. Insulin receptors are translocated to the nucleus via an internalization process that begins upon ligand binding to receptors on the cell membrane (21-23). Newly translocated insulin receptor retains its tyrosine kinase activity (22), which suggests that it is able to phosphorylate tyrosine sites on nuclear proteins. The present research is based on a model used to observe accumulation of the insulin receptor in the nucleus following insulin stimulation *in vivo*. Using this model, it was demonstrated that active insulin receptors accumulate in the nucleus within 15 min of insulin stimulation and that insulin induction of ME and GAPDH gene expression occur concomitantly with the time course for insulin receptor translocation to the nucleus. A decrease in the phosphorylation of a DNA-binding protein was observed within the time course of insulin receptor appearance in the nucleus. Although active insulin receptor accumulated in the nucleus of glucose-treated mice, the changes that occurred in nuclear processes may have been a result of cytoplasmic signaling initiated by insulin receptor activation of IRS-1. The objective of this study was to determine if changes in nuclear protein phosphorylation and gene expression induced by insulin stimulation were initiated upon translocation of insulin receptor to the hepatocyte nucleus in the absence of cytoplasmic signaling. Nuclei, taken from hepatocytes of mice fasted for 24 hours, were incubated with purified insulin receptor. The effect of direct incubation of insulin receptor on the phosphorylation of nuclear proteins and the affinity of malic enzyme IRE-binding proteins was determined.

## MATERIALS AND METHODS

### Animals Care and Treatments

Male C57 Black 6 mice (Jackson Laboratories, Bar Harbour, Maine, U.S.A.), were housed in groups of four to six in polypropylene cages in a humidity and temperature controlled room with a 12 hr light/dark cycle. Mice were given free access to a standard laboratory chow from the age of weaning (Laboratory Standard Rodent Diet 5001, PMI Feeds, Inc., MO, USA. At eight weeks of age, mice were fasted for 24 hours beginning at 9:00 am preceeding the experimental day. Mice treated with glucose were then given an oral gavage of 3 g glucose / kg body weight. At various times following the gavage animals were anestitized with halothane and then blood was removed by cardiac puncture for glucose and insulin determinations. Animals were terminated by cervical dislocation.

### Nuclei Isolation

Liver was immediately excised and minced in homogenization buffer containing 250 mM glucose, 50 mM Tris-HCl, 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl flouride (PMSF), 10 μM sodium orthovanadate, 1 mM $MgCl_2$, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.0044 Trypsin Inhibitory Units (TIU)/ml aprotinin at pH 7.5. Nuclei were isolated as described by Blobel and Potter (27), with the following modifications. Tissue was homogenized using a Potter-Evelhjem homogenizer with a motor driven teflon pestle and then centrifuged at 1000 x g for 20 min using a Beckman JA20 rotor (Paloalto, California, USA). Pellets were resuspended in homogenization buffer and then the sucrose concentration of the homogenate was raised to 1.6 M using homogenization buffer containing 2.4 M sucrose. The homogenate was centrifuged at 124 000 x g for 70 min using a Beckman SW 28 rotor. Nuclei pellets were resuspended using a homogenizer in nuclei isolation buffer containing 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2.5 mM KCl, 1 mM benzamidine-HCl, 1 mM PMSF, 10 μM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.0044 TIU/ml aprotinin. One volume of nuclei isolation buffer containing 2.2 M sucrose, was added to the nuclear suspension. This mixture was layered over five ml of the 2.2 M sucrose containing buffer and centrifuged at 124 000 x g for 60 min using a SW28 rotor. Nuclear pellets were resuspended in nuclear isolation buffer, mixed with one volume of 2.2 M sucrose containing buffer, layered over 2.2 M sucrose containing buffer and recentrifuged at 124 000 x g for 60 min. Final nuclei pellets were suspended in nuclei isolation buffer. To check purity of isolated nuclei, nuclei suspensions were inspected under the light microscope after staining with 0.2 % (v/v) methylene blue and ethidium bromide (100 μg/ml) at 400 x magnification.

### *Preparation of DNA-Binding Nuclear Proteins*

DNA binding proteins were prepared from nuclear extract as described (28). The purified nuclear pellet was resuspended in buffer containing 50 mM Tris-HCl pH 7.4, 250 mM sucrose, 5 mM $MgSO_4$, 1 mM PMSF, 0.0015 TIU/ml aprotinin, and 5 μM leupeptin. DNase 1 and RNase A were added to suspensions to 250 μg/ml final concentration each. After 1 h of incubation, the nuclei were sedimented at 5000 x g for 10 min. The supernatant (Sup1), containing soluble nuclear proteins and proteins weakly associated with DNA was collected. The pellet was resuspended in a buffer containing

10 mM Tris-HCl pH 7.4, 0.2 mM $MgSO_4$, 1 mM PMSF, and 0.0015 TIU/ml aprotinin. This buffer containing 2.0 M NaCl was then slowly added to a final NaCl concentration of 1.6 M. After 15 min incubation, the residual structures were sedimented at 5000 x g for 20 min. The supernatant (Sup2), which contained tightly bound DNA proteins, was collected and stored at -70$^0$C. Protein content was determined (29)

***Direct Phosphorylation of Isolated DNA-Binding Proteins by Purified Insulin Receptor***

Isolated DNA-binding proteins (Sup2) were prepared from the nuclei of hepatocytes taken from mice fasted for 24 h. 50 µg protein from each sample was incubated with reaction mixture containing 60 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 230 mM NaCl, 1 mM PMSF, 0.4 mM adenosine triphosphate, 8 mM $MnCl_2$, 2mM dithiothreitol, and 2 µg/ml leupeptin. To begin the labeling of DNA binding proteins [$\gamma^{32}$P]-ATP (2 µCi) was added and the mixture incubated for 15 min at room temperature. At the same time 2 µg purified insulin receptor preparation in reaction mixture without dithiothreitol was activated using 10 nM insulin for 15 min at room temp. To the labeled DNA binding proteins that had been labeled with [$\gamma^{32}$P]-ATP, 0.2 µg of activated receptor was added. The reaction was carried out for 0, 10, 15, and 60 min and stopped by adding 4X Laemelli sample buffer (30) and then boiled for 5 min. Controls for each time were prepared by incubating an equivalent DNA-binding protein extract for the same amount of time with [$\gamma^{32}$P]-ATP but without activated insulin receptor added. Samples were loaded onto a 7.5 % (w/v) polyacrylamide gel and subjected to SDS-PAGE at 25 mA. The gel was stained with 0.025 % (w/v) Coomassie brilliant blue R250 (BioRad Laboratories, Hercules, California, USA), 40 % (v/v) methanol, and 10 % (v/v) acetic acid and vacuum dried. The $^{32}$P-labeled DNA binding proteins were visualized by autoradiography and quantitated by densitometry using the GS-670 Imaging Densitometer (BioRad).

### *Gel Shift Assay*

Nuclear extracts were prepared from liver as described above with the exception that final pellets were suspended in 25 mM N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.6, 40 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 10 % (v/v) glycerol, and stored at -70$^0$C until use. The oligonucleotides used in the assay (5'-CCCGCCCCCGCCTCCTCGCA-3' and its complement), corresponded to the sequences -175 to -156 of the promoter region of the malic enzyme gene. The underlined sequence, CGCCTC, within the oligonucleotide has previously been described as an insulin responsive element within the malic enzyme gene promotor (5). The oligonucleotides (50 µg each) were labeled by incubating the oligonucleotides with 150 µCi of [$\gamma$-$^{32}$P ]-ATP, and T4 polynucleotide kinase (10 units) (GIBCO BRL Life Technologies Gaithersburg, MD USA), for 1 h at 37$^0$C. Labeled DNA was purified by adding 0.2 volumes of 5 M ammonium acetate and two volumes of 100 % ethanol. DNA was allowed to precipitate overnight at -20$^0$C. The DNA-labeled pellet was collected by centrifugation at 14 000 x g for 15 min (Jouan Microcentrifuge, Winchester VA, USA), and dissolved in 50 µl water. 2 pmol of labeled probe (20 000 dpm) was used per reaction.

The binding reaction was carried out with 5 µg of nuclear extract in a binding reaction mixture containing 40 mM HEPES, pH 7.9, 200 mM KCl, 0.5 mM dithiothreitol, 0.2 mM

EDTA, 10 % (v/v) glycerol, and 3 μg Sonicated Calf Thymus DNA (Pharmacia Biotech. Inc.), and was incubated for 15 min on ice. In competition experiments, unlabeled oligonucleotides were added in excess (160x) or 30 ng of EBNA-1 DNA, an 82-mer unrelated DNA was added (Pharmacia Biotech. Inc.). 2 pmol of labeled probe was added to the mixture and incubated for 30 min at room temp. 2 μl of loading dye containing 250 mM Tris-HCl (pH 7.5), 0.2 % (v/v) bromophenol blue, 0.2 % (v/v) xylene cyanol, and 40 % (v/v) glycerol was added to the mixture. Free DNA and DNA-protein complexed were resolved on native 5 % (w/v) polyacrylamide gels in 0.5x TBE (1X TBE is 90 mM Tris, 90 mM boric acid and 1 mM EDTA pH 8.0) run at 20 mA before being vacuum dried and exposed to a X-ray film at -70$^0$C. Bands were quantified using the GS-670 Imaging Densitomer from BioRad Laboratories, Hercules, CA, USA.

***Direct Incubation of Nuclei with Purified Insulin Receptor Prior to Gel Shift Assay***

Experiments were designed to evaluate nuclear protein binding affinity to ME-IRE following incubation for varying lengths of time with purified insulin receptor. Purified insulin receptor (5 μg) was activated in reaction mixture containing 60 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 230 mM NaCl, 1 mM PMSF, 0.4 mM adenosine triphosphate, 8 mM $MnCl_2$, and 2 μg/ml leupeptin, by adding 10 nM insulin and incubating for 15 min at room temp. Activated insulin receptor (0.25 μg) was then added to nuclear extracts for 10, 15 or 30 min incubation at room temp. Nuclear extracts were then incubated in binding mixture containing 40 mM HEPES, pH 7.9, 200 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA, 10 % (v/v) glycerol, and 3 μg Sonicated Calf Thymus DNA, for 15 min on ice. The gel shift assay was continued as described above.

Experiments were designed to evaluate nuclear protein binding affinity to ME-IRE after incubation with varying doses of purified insulin receptor. Activated insulin receptor was incubated with nuclear extracts to a final concentration of 0.0063, 0.013, 0.025, 0.05 µg/µl for 15 min at room temp. Bovine serum albumin (Fraction V, Sigma) was substituted for insulin receptor so that the protein added to nuclear extracts totaled 0.05 µg/ml. Non-activated insulin receptor was prepared without insulin added to incubation mixture and was used in place of insulin receptor to serve as a control. Nuclear extracts were then incubated in binding mixture and gel shift assay continued as described above.

***Purification of the Insulin receptor***

Insulin receptor was purified from plasma membrane isolated from liver of male Sprague Dawley rats. Animals were treated as described for mice. Prior to an experiment day, animals were fasted for 12 hours, beginning at 8:00 p.m. and ending at 8:00 a.m. on the day of the experiment. To collect liver tissue, rats were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation. At the time of sacrifice animals were eleven weeks of age, weighed 440 ± 17 g and liver tissue weighed 18.9 ± 0.7g (n=24).

Plasma membrane was prepared according to established procedures (31) and modified as follows. Following sacrifice, livers were extracted and placed in ice-cold homogenization medium containing 0.25 M sucrose, 5 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM PMSF, 1 mM benzamidine-HCl, 1 µg/ml leupeptin, 1µg/ml pepstatin, 0.0044 trypsin inhibitor units/ml (TIU/ml) aprotinin. Livers were minced and homogenized in 5 ml/g liver using a Potter-Elvehjem homogenizer. Homogenate was centrifuged at 1450 x $g_{max}$ for 20 min in a JA14 rotor (Beckman), to pellet the crude nuclear fraction. The supernatant was centrifuged at 8 700 x $g_{max}$ for 20 min to pellet mitochondria. The remaining supernatant was spun at 105 000 x $g_{max}$ for 30 minutes in a Ti45 rotor to pellet plasma membranes. Plasma membranes were then purified by resuspending the pellet in buffer containing 0.25 M sucrose, 5 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM PMSF, 1 benzamidine-HCl, 1 µg/ml leupeptin, 1 µg/ml pepstatin A pH 7.5, and then adding this buffer (1.04 volumes) containing 2.4 M sucrose so that the final concentration of the homogenate was 1.35 M. The suspension was aliquoted into centrifuge tubes and overlayed with 0.3 volumes of the 0.25 M sucrose-containing buffer. Tubes were centrifuged at 231 000 x $g_{av}$ for 30 min using a T170.1 rotor (Beckman), and plasma membranes were collected at the interface.

Insulin receptor was purified from plasma membrane as described (32) with modifications. Plasma membranes were solubilized in a buffer containing 50 mM Tris-HCl pH 7.5, 1 mM PMSF, 1 mM benzamidine-HCl, 1 µg/ml leupeptin, 100 µg/ml pepstatin A, 10 TIU/ml aprotinin, and 2 % (v/v) Triton X-100 (Sigma) for 2 h at 4$^0$C. Insoluble proteins were precipitated by centrifugation at 100 000 x $g_{av}$ for 1 hr in a SW28 rotor (Beckman). Supernatant was supplemented with 0.09 % (w/v) $MgCl_2$ and applied to Triticum Vulgaris (Wheat germ)-agarose (Sigma) preequilibrated in 50 mM HEPES pH 7.6 , 1 mM PMSF, 1 mM benzamidine-HCl, and 150 mM NaCl, and mixed gently overnight at 4$^0$C. The gel slurry was washed with 50 mM HEPES, 1 mM PMSF, 1 mM benzamidine, 150 mM NaCl, 10 mM $MgSO_4$, 0.1 % (v/v) Triton X-100, pH 6.0 (20 volumes), and 50 mM HEPES pH 7.6, 1 mM PMSF, 1 mM benzamidine-HCl, 150 mM NaCl, 0.1 % (v/v) Triton X-100, pH 7.6 (10 volumes). Insulin receptor bound to the column was eluted with 50 mM HEPES, 1 mM PMSF, 1 mM benzamidine-HCl,, 150 mM NaCl, 0.1 % (v/v) Triton X-100, 0.5 TIU/ml aprotinin, 20 uM pepstatin A, 20 uM leupeptin, and 0.3 M N-acetyl-D-glucosamine, pH 7.6. The eluted glycoprotein fraction was supplemented with 0.09 % (w/v) $MgCl_2$ and mixed overnight at $4^0$C with Insulin-Agarose (Sigma) that had been preequilibrated with 50 mMTris-HCl, 0.1 mM PMSF, 0.1 % (v/v) Triton X-100 pH 7.5. The gel slurry was poured into a glass column and washed with 50 mM Tris-HCl, 0.1 mM PMSF, 0.1 % (v/v) Triton X-100, and 1 M NaCl, pH 7.5 (100 volumes). Insulin receptor bound to the column was eluted with 0.05 M sodium acetate, pH 5.0 (2 volumes). Fractions (1 ml) were collected into tubes containing 0.1 ml 1 M HEPES, 0.1 % (v/v) Triton X-100, 0.1 mM PMSF, and 20 mM ethyleneglycoltetraacetate, pH 7.8. Insulin receptor was assayed during various steps in the purification using insulin binding assays (32,33), and by western blotting assays using anti-insulin receptor antibody (Transduction Laboratories, Lexington, Kentucky, USA). Purified insulin receptor was stored in 50 % (v/v) glycerol at $-70^0$C. For more details about purification of insulin receptor see **Appendix II, III and IV.**

### *Statistical Evaluation*

For analysis of nuclear protein phosphorylation upon incubation with insulin receptor, three experiments were carried out, each on different days. In each experiment three different animals were examined for each time of incubation with insulin receptor (n=9 for each time assessed). For the effects of oral glucose gavage on IRE-binding, n=6 for the 0 and 15 min times and n=2 for the 180 min time following oral glucose gavage. For the effects of insulin receptor incubation on IRE-binding, 5 animals were used for each dose of insulin receptor assessed, and one animal was used for the time-response experiment. The differences between selected times were assessed using analysis of variance (SAS Version 6.11 SAS Inc., Cary, NC, USA). In some cases as indicated, statistical differences between comparisons were assessed using Students *t*-tests ($p<0.05$) (34,35).

## *RESULTS*

### ***Incorporation of $P^{32}$ into DNA-Binding Proteins After Incubation with insulin receptor***

DNA-binding proteins prepared from isolated hepatocyte nuclei taken from mice that had been fasted for 24 h were labeled with [$\gamma^{32}$P]-ATP for 15 min and then incubated for a period of time in the presence or absence of purified insulin receptor. The amount of labeled phosphate incorporated into several DNA-binding proteins was observed to increase with time whether or not insulin receptor was present **(Figure 5.1)**. The labeling of nuclear protein with [$\gamma^{32}$P]-ATP after the addition of insulin receptor (time 0) was quantified. Accumulation of $^{32}$P-phosphate into protein peaked at 15 min of incubation and had decreased by 60 min of incubation with [$\gamma^{32}$P]-ATP. Insulin receptor added to nuclear proteins decreased the incorporation of $^{32}$P-phosphate into two DNA-binding proteins. One protein (p37) migrated at 37 KDa **(Figure 5.2)** and the other (p30) at 30 KDa **(Figure 5.3)**. At 15 min of incubation with insulin receptor, the amount of $^{32}$P-phosphate observed in p37 and in p30 was reduced by 78 % and 73 % respectively, compared to the levels of $^{32}$P-phosphate observed in the absence of insulin receptor.

### *Transcription Factor Binding to Malic Enzyme IRE*

The effect on oral glucose administration on the binding affinity of proteins isolated from hepatocyte nuclei to malic enzyme-IRE was determined. Results from gel-shift assays suggest that two different nuclear proteins bound to the malic enzyme IRE **(Figure 5.4 lane B)**. The binding of nuclear proteins to labeled IRE probe was specific; it was abolished in the presence of excess unlabeled malic enzyme-IRE **(Figure 5.4 lane C)** but was retained in the presence of an unrelated oligonucleotide **(Figure 5.4 lane NC)**. Nuclear protein binding to the IRE within the malic enzyme gene increased within 15 min ($p<0.05$) and decreased to below binding levels at basal conditions by 180 min following oral glucose gavage **(Figure 5.5)**.

### *Transcription Factor Binding to Malic Enzyme IRE Following Incubation with Insulin Receptor*

The effect of insulin receptor on the binding affinity of proteins to the malic enzyme promoter was accessed by incubating purified insulin receptor with preparations of nuclear proteins isolated from the liver of mice fasted for 24 hours. A time course experiment demonstrated that incubation of insulin receptor with nuclear preparations prior to the addition of labeled probe, increased the protein-DNA complex over that observed in the absence of insulin receptor **(Figure 5.6)**. Increasing doses of insulin receptor were added to nuclear preparations isolated from the liver of fasted mice prior to the incubation of nuclear proteins with labeled probe. The complex formed between labeled IRE probe and proteins present in the nuclear preparations was increased with increasing amounts of insulin receptor added **(Figure 5.7)**. Dose-response studies demonstrated that effects of insulin receptor were half-maximal at a dose of 0.0063 μg/μl and maximal at about 0.025 μg/μl.

## *DISCUSSION*

Incubation of protein isolated from the hepatocyte cell nucleus with purified insulin receptor was designed to simulate conditions that would occur upon translocation of receptor to the nucleus. Hepatocyte nuclei were isolated from animals that had been fasted for 24 h. Under these conditions serum insulin concentrations were 100 fold below the Km of receptor phosphorylation (2 nM) (36). Therefore it could be assumed that insulin signaling to the nucleus via intracellular mediators could not be responsible for the effects observed. Addition of [$\gamma^{32}$P]-ATP caused the labeling of DNA-binding proteins with $^{32}$P-phosphate, suggesting that one or more kinases were constitutively active in the nucleus. Insulin receptor rapidly attenuated phosphate incorporation into two DNA-binding proteins, a 37 KDa protein and a 30 KDa protein. In a previous study **(Chapter 4)**, the tyrosine phosphorylation state of a 30 KDa DNA-binding protein was observed to be decreased within 15 min of stimulation with insulin *in vivo*. Thus insulin receptor, in the absence of known cytosolic mediators of insulin signaling, was able to produce a similar dephophorylation of a nuclear protein as was observed following stimulation with insulin *in vivo*. This evidence suggests that insulin receptor may directly generate insulin signaling in the nucleus.

Insulin stimulation of the phosphorylation (7,8,37,38) and dephosphorylation (39,40) of several nuclear proteins and transcription factors has been demonstrated. In these studies, insulin regulation of protein phosphorylation was demonstrated upon stimulation of the intact cell with insulin. Changes in phosphorylation state of these nuclear proteins (7,8,37-40) were not observed in the present study following treatment of cell nuclei with insulin receptor. Therefore insulin-stimulation of cells versus insulin receptor treatment of nuclei produce different effects on the phosphorylation state of DNA-binding proteins. This evidence suggests that the mechanisms used in insulin signal transduction to the nucleus when derived from the cell surface differ from signal transduction to the nucleus when derived by direct interaction of nuclear-translocated insulin receptor with nuclear proteins.

It is unclear how the insulin receptor, which itself is a tyrosine kinase, is able to inhibit incorporation of $^{32}$-P-phosphate into the two DNA-binding phosphoproteins observed in the present study. These proteins may be substrates of a kinase the activity of which is inhibited by insulin. The 30 KDa or the 37 KDa phosphoprotein may be substrates of a nuclear phosphatase the activity of which is increased by insulin stimulation. Insulin action on nuclear phosphatases has been reported (39,40). In one study (39), glucose treatment of mouse adipocytes induced the activation of a 38 KDa nuclear phosphatase. Future studies are needed to determine if this protein is an endogenous substrate of the nuclear insulin receptor tyrosine kinase. Addition of insulin to highly purified liver nuclei taken from diabetic rats resulted in a decrease in the phosphorylation of $^{32}$-P-phosphate into nuclear proteins (40). The physiological condition, fed or fasted, of the animals used in this study was not described, therefore it is not possible to assess if insulin receptor translocation to the nucleus may have occurred. It is possible that in this study insulin receptors in the nucleus were involved in regulating the activity nuclear phosphatases in response to insulin treatment (40).

Transcription factors that bind to the insulin responsive sequence of the malic enzyme promotor have been previously identified (5). Retardation of the malic enzyme IRE-probe by two nuclear proteins was observed in this study as was reported in (5). Insulin stimulation via oral glucose gavage induced an increase in binding of transcription factors to the ME-IRE compared to the binding observed under fasting conditions within 15 min after glucose administration. Treatment of nuclear proteins from animals in the fasted condition with insulin receptor caused increased binding of transcription factors to probe. The effect of insulin receptor on the binding affinity of transcription factors was dose-dependent and saturable indicating that the effect was specifically mediated by the receptor and that the effect was limited by the amount of transcription factors available in the nuclear preparation. Insulin receptor induced an increase in binding affinity of transcription factors and a concomitant dephosphorylation of nuclear proteins. Taken together these findings suggest that it is an increase in phosphatase activity and a resulting dephosphorylation of transcription factors that causes an increase in binding to the IRE within the malic enzyme gene. Transcription factor dephosphorylation resulting in an increase in DNA-binding activity is used as a mechanism for regulation of gene transcription (6). In one study using adipocytes, an increase in binding of the transcription factor Sp1 to the acetyl Co-A carboxylase gene IRE was attributed to dephosphorylation of Sp1 that occurred following glucose treatment (39). The core sequence motif CGCCTC within the malic enzyme gene shares some homology to the IRE within the GAPDH (2) and glucagon (34) genes, and this motif may be recognized by Sp1 (41). As Sp1 consists of two species of 95 KDa and 105 KDa (42), it is possible that it is the binding of Sp1 to the ME-IRE that produces the two shifted bands observed in the gel-shift assay (43).

The results of the present study suggest that insulin receptor in the isolated nucleus alters the phosphorylation state of DNA-binding proteins and increase binding of transcription factors to the ME-IRE. The model of insulin signal transduction proposed in this chapter is described in a schematic (**Figure 5.8**). As these responses were observed in the absence of cytosolic mediators within the insulin signaling cascade, the present findings suggest a novel mechanism by which insulin regulates processes involved in ME gene expression.

## *REFERENCES*

1. O'Brien, R. M., and Granner, D.K. Regulation of gene expression by insulin. (1996). Physiol. Rev.,76(4):1109-1161.

2. Alexander, M.C., Lomanto, M., Nasrin, N., and Ramaika, C. (1988) Proc. Natl. Acad. Sci. USA,85:5092-5096.

3. Moustaid, N., Beyer, R.S., and Sul, H.S. Identification of an insulin-response element in the fatty acid synthase promotor. (1994) J. Biol. Chem.,269(8):5629-5634.

4. O'Brien, R.M., Lucas, P.C., Forest, C.D., Magnuson, M.A., and Granner, D.K. (1990) Science,249:533-537.

5. Garcia-Jimenez, C., Benito, B., Jolin, T., and Santisteban, P. Insulin regulation of malic enzyme gen expression in rat liver: evidence for nuclear proteins that bind to two putative insulin response elements. (1994) Mol. Endocrinol..8:1361-1369.

6. Hunter, T., and Karin, M. The regulation of transcription by phosphorylation. (1992) Cell,70:375-387.

7. Csermely, P., and Kahn, C.R. Insulin induces the phosphorylation of DNA-binding nuclear proteins including lamins in 3T3-F442A. (1992) Biochem.,31:9940-9946.

8. Csermely, P., Schnaider, T., Cheatham, B., Olson, M.O.J., and Kahn, C.R. Insulin induces the phosphorylation of nucleolin. A possible mechanism of insulin induced mRNA efflux from nuclei. (1993) J. Biol. Chem.,268(13):9747-9752.

9. Kim, S-J, and Kahn, C.R. Insulin stimulates phosphorylation of c-Jun, c-Fos, and Fos-related proteins in cultured adipocytes. (1994) J. Biol. Chem.,269(16):11887-11892.

10. Kasuga, M., Karlsson, F.A., and Kahn, C.R. Insulin stimulates the phsphorylation of the 95,000-dalton subunit of its own receptor. (1982) Science,215:185-186.

11. Sun, X,-J, Rothenberg, P., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J., and White, M.F. The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. (1991) Nature Lond.,352:73-77.

12. Kahn, C.R., White, M.F., Shoelson, S.E., Backer, J.M., Araki, E., Cheatham, B., Csermely, P., Folli, F., Goldstein, B.J., Huertas, P., Rothenberg, P.L., Saad, M.J.A., Siddle, K., Sun, X-J., Wilden, P.A., Yamada, K., and Kahn, S.A. The insulin receptor and its substrate: molecular determinants of early events in insulin action. (1993) Rec. Prog. Horm. Res.,48:291-229.

13. Araki, E.M., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, B., Johnson, R.S., and Kahn, C.R. Alternative pathway of insulin signaling in mice with targeted disruption of the IRE-gene. (1994) Nature Lond.,372:186-190.

14. Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakuda, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., Sekihara, S., Yoshioka, S., Horikoshi, H., Furuta, Y., I-Kawa, Y., Kasuga, M., Yazaki, Y., and Aizawa, S. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. (1994) Nature Lond.,372:182-186.

15. Weswick, J.K., Cox, A.D., Der, C.J., Cobb, M.H., Hibi, M., Karin, M. and Brewer, D.A., Oncogenic ras activates c-Jun via a separate pathway from the activation of extracellular signal-regulated kinases. (1994) Proc. Natl. Acad. Sci. USA,91:6030-6034.

16. Ouyang, L., Jacob, K.K., and Stanley, F.M. GABP mediates insulin-increased prolactin gene transcription. (1996) J. Biol. Chem.,271(18):10425-10428.

17. Gabbay, R.A., Sutherland, C., Gnudi, L., Kahn, B.B., O'Brien, R.M., Granner, D.K., and Flier, J.S. Insulin regulation of phosphoenolpyruvate carboxykinase gene expression does not require activation ot the Ras/Mitogen activated protein kinase signaling pathway. (1996) J. Biol. Chem.,271(4):1890-1897.

18. Owawa, H., Sutherland, C., Robey, R.B., Printz, R.L., and Granner, D.K. Analysis of the signaling pathway involved in the regulation of hexokinase II gene transcription by insulin. (1996) J. Biol. Chem.,271(28):16690-16694.

19. Sutherland, C., O'Brien, R.M., and Granner, D.K. Phosphatidylinositol 3-kinase, but not p70/p85 ribosomal S6 protein kinase, is responsible for the regulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression by insulin. Dissociation of signaling pathways for insulin and phorbol ester regulation of PEPCK gene expression. (1995) J. Biol. Chem.,270(26):15501-15506.

20. Yang, S.H., and Dickson, A.J. Inhibitors of signaling identify differential control processes responsible for selective effects of insulin on the expression of phosphoenolpyruvate carboxykinase and gene 33 in rat H4 hepatoma cells. (1995) Biochem. J.,310(2):375-378.

21. Smith, R.M., and Jarett, L. Ultrastructural evidence for the accumulation of insulin in nuclei of intact 3t3-L1 adipocytes by an insulin-receptor mediated process. (1987) Proc., Natl., Acad., Sci., U.S.A., 84:459-463.

22. Kim, S.-J., and Kahn, C.R. Insulin induces rapid accumulation of insulin receptors and increases tyrosine kinase activity in the nucleus of cultures adipocytes. (1993) J. Cell. Physiol., 157:217-228.

23. Podlecki, D.A., Smith, R.M., Kao, M., Tsai, P., Huecksteadt, T., Brandenburg, D., Sasher, R.S., Jarett, L., and Olefsky, J.M. Nuclear translocation of the insulin receptor. A possible mediator of insulin's long term effects. (1987) J. Biol. Chem., 292:3362-3363.

24. Lobie, P.E., Wood, T.J.J., Chen, C.M., Waters, M.J., and Norstedt, G. Nuclear translocation of the growth hormone receptors. (1994) J. Biol. Chem.,269(50):31735-31746.

25. Jiang, L-W., and Schindler, M. Nucleocytoplasmic transport is enhanced concomitant with nuclear accumulation of epidermal growth factor (EGF) binding activity in both 3T3-1 and EGF receptor reconstituted NR-6 Fibroblasts. (1990) J. Cell. Biol.,110:559-568.

26. Fraser, R.A., and Harvey, S. Ubiquitous distribution of growth hormone receptors and/or binding protein in adenohypophyseal tissue. (1992) Endocrinol.,130:3593-3600.

27. Blobel, G., and Potter, V.R. Nuclei from rat liver: isolation method that combines purity wth high yield. (1966) Science, 154:1662-1665.

28. Jaumot, M., Agell, N., and Bachs, O. Putative nuclear cdk2 substrates in normal and transformed cells. (1996) Biochem. Biophys. Res. Comm.,219(6):560-564.

29. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the Folin phenol reagent. (1951) J. Biol. Chem., 193:265-275.

30. Laemmli, U.K. (1970) Nature (London),227:680-685.Helmerhorst, E., and Nakhoul, A.-M. Novel affinity support prepared by crosslinking purified insulin receptors to agarose. (1993) Biochem. Biophys. Res. Comm., 195(1):237-241.

31. Aronson, N.N.Jr., and Touster, O. Isolation of rat liver plasma membrane fragments in isotonic sucrose. (1974) Methods in Enzymology, 31:90-102.

32. Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y., and Itakura, K. Purification of insulin receptor with full binding activity. (1983) J. Biol. Chem., 258(8):5045-5049.

33. Desbuquois, B., and Aurbach, G.D. Use of polyethylene glycol to separate free and antibody bound peptide hormones in radioimmunoassays. (1971) J. Clin. Endocr., 33:732-738.

34. Duncan, D.B. Multiple range and multiple *F* tests. (1955) Biom.,11:1-42.

35. Steel, R.G.D., and Torrie, J.H. (1980) in Principles and Procedures of Statistics, 2nd edn., McGraw-Hill Book Co., Ch. 8-9, New York, NY, USA.

36. Balloti, R., Kawalsky, A., White, M.F., Le Marchand-Brustel, Y., and Van-Obberghen, E. Insulin stimulates tyrosine phosphorylation of its receptor β-subunit in intact hepatocytes. (1987) Biochem. J.,24:91-

37. Reucsh, J.E.B., Hsieh, P., Bhuripanyo, P., Carel, K., Leitner, J.W., Olefsky, J.M., and Draznin, B. Insulin inhibits nuclear phosphatase activity: Requirement for the C-terminal domain of the insulin receptor. (1995) Endocrinol., 136:2464-2469.

38. Csermely, P., and Kahn, C.R. Insulin induces the phosphorylation of DNA-binding nuclear proteins including lamins in 3T3-F442A. (1992) Biochem.,31:9940-9946.

39. Daniel, S., Zhang, S., DePaoli-Roach, A.A., and Kim, K-H. Dephosphorylation of Sp1 by protein phosphatase 1 is involved in the glucose-mediated activation of the acetyl-coA carboxylase gene. (1996) J. Biol. Chem., 271(25):14692-14697.

40. Purrello, F., Burnham, D.B., and Goldfine, I.D. Insulin regulation of protein phosphorylation in isolated rat liver nuclear envelopes: Potential relationship to mRNA metabolism. (1983) Proc. Natl. Acad. Sci. USA, 80:1189-1193.

41. Philippe, J. Insulin regulation of the glucagon gene is mediated by insulin-responsive element. (1991) Proc. Natl. Acad. Sci. USA,88:7724-7727.

42. Kadonaga, J.T., and Tjian, R. Promoter-specific activation of RNA-polymerase II transcription by Sp1. (1986) Trends Biochem.,11:20-23.

43. Kadonaga, J.T., Courey, A.J., Ladika, J., and Tjian, R. Distinct regions of Sp1 modulate DNA-binding activity and transcriptional activation. (1988) Science,242:1566-1569.

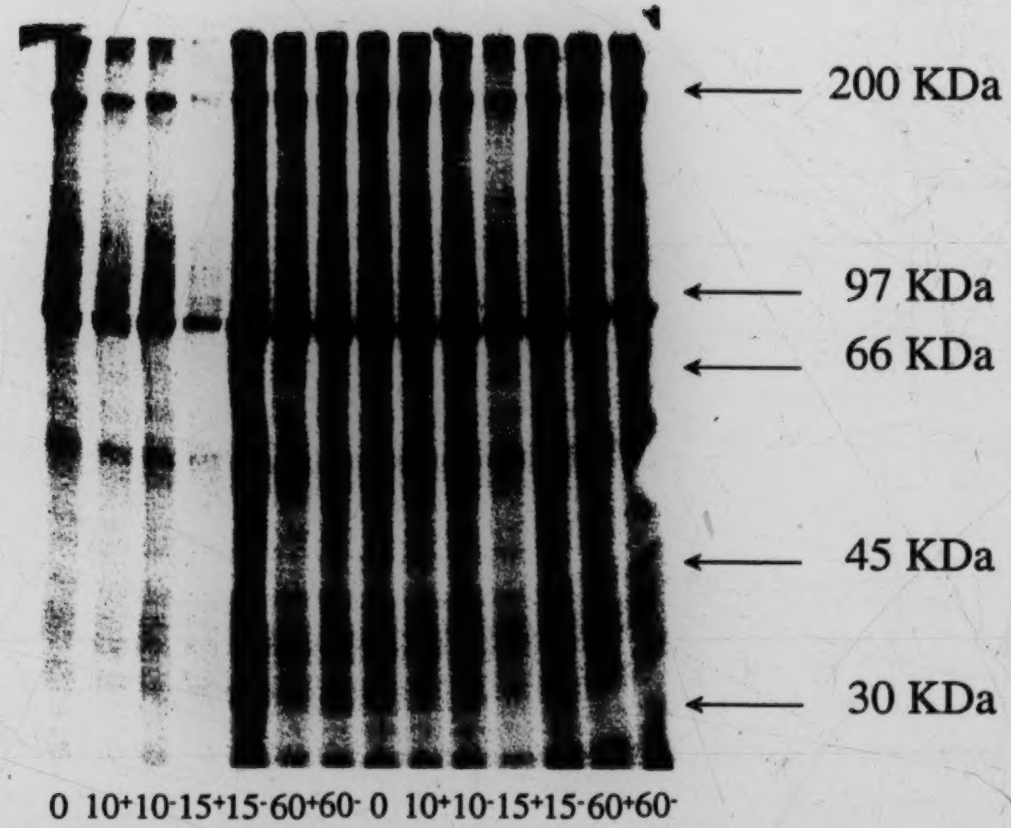


**Figure 5.1 Phosphorylation of DNA-binding proteins following incubation with $P^{32}$ and purified insulin receptor.**

Nuclei were isolated from hepatocytes taken from mice fasted for 24 h. DNA binding proteins were prepared from nuclei. Proteins were labeled with $[\gamma^{-32}P]$-ATP for 20 min before the addition of 0.2 μg of activated insulin receptor. The reaction was carried out for 0, 10, 15, and 60 min and stopped by adding sample buffer and boiling for 5 min. Controls for each time contained no insulin receptor. Proteins were subjected to SDS-PAGE followed by autoradiography to detect radioactivity incorporated into protein. Bands were quantitated by densitometry. Arrows indicate location of molecular weight standards. Lanes are labeled with incubation time and with (+) or without (-) insulin receptor added.

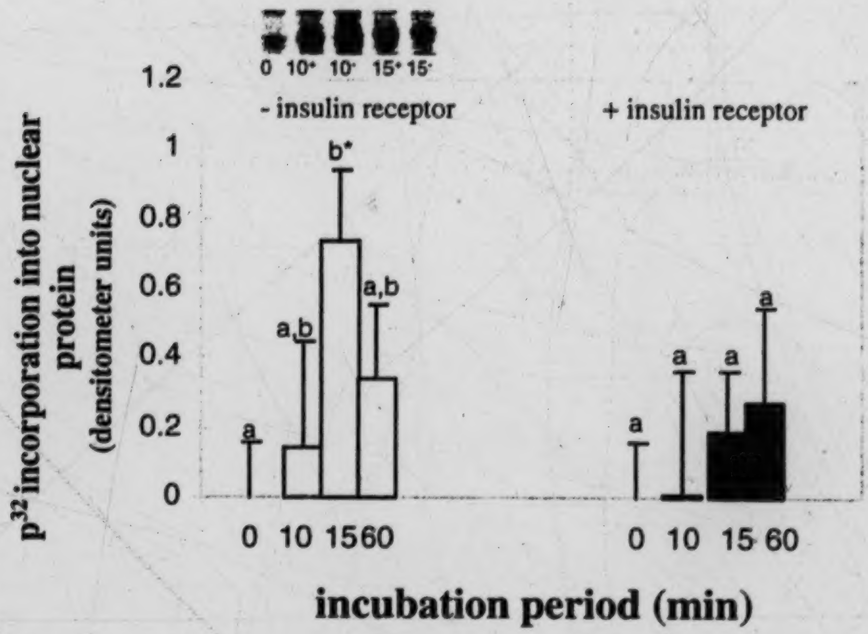


**Figure 5.2 Incorporation of $^{32}$P-phophate into 37 KDa nuclear protein following incubation with insulin receptor**

Nuclei were isolated from hepatocytes taken from mice were fasted for 24 h. DNA binding proteins were prepared from nuclei. Proteins were labeled with [γ $^{32}$P]-ATP for 20 min before the addition of 0.2 μg of activated insulin receptor. The reaction was carried out for 0, 10, 15, and 60 min and stopped by adding sample buffer and boiling for 5 min. Controls for each time contained no insulin receptor. Proteins were subjected to SDS-PAGE followed by autoradiography to detect radioactivity incorporated into protein. Bands were quantitated by densitometry and values are expressed as a change from basal in densitometer units (0 =0.87 ± 0.07 ). Comparisons of the effect of time within each treatment with (+) or without (-) insulin receptor added were assessed using ANOVA. Values labeled with different letter are significantly different ($p<0.05$). Comparisons of the effect of treatment for each time were made using Student *t*-test ($p<0.05$). Values without insulin receptor treatment [(-) ir] labeled with (*) are significantly different from values at the same time with insulin receptor treatment[(+)ir]. N = 9 for each timepoint

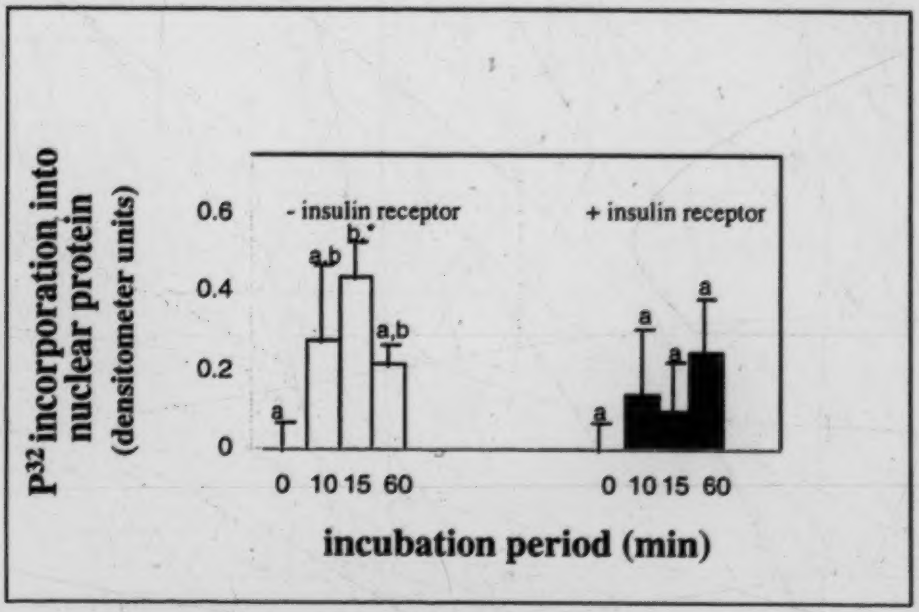


**Figure 5.3 Incorporation of $^{32}$P-phophate into a 30 KDa nuclear protein following incubation with insulin receptor**

Nuclei were isolated from hepatocytes taken from mice were fasted for 24 h. DNA binding proteins were prepared from nuclei. Proteins were labeled with [$\gamma$ $^{32}$P]-ATP for 20 min before the addition of 0.2 µg of activated insulin receptor. The reaction was carried out for 0, 10, 15, and 60 min and stopped by adding sample buffer and boiling for 5 min. Controls for each time contained no insulin receptor. Proteins were subjected to SDS-PAGE followed by autoradiography to detect radioactivity incorporated into protein. Bands were quantitated by densitometry and values are expressed as a change from basal in densitometer units (0 = 0.95 ±0.16). Comparisons of the effect of time within each treatment with (+) or without (-) insulin receptor added were assessed using ANOVA. Values labeled with different letter are significantly different ($p<0.05$). Comparisons of the effect of treatment for each time were made using Student *t*-test ($p<0.05$). Values without insulin receptor treatment [(-) ir] labeled with (*) are significantly different from values at the same time with insulin receptor treatment[(+)ir]. N = 9 for each timepoint

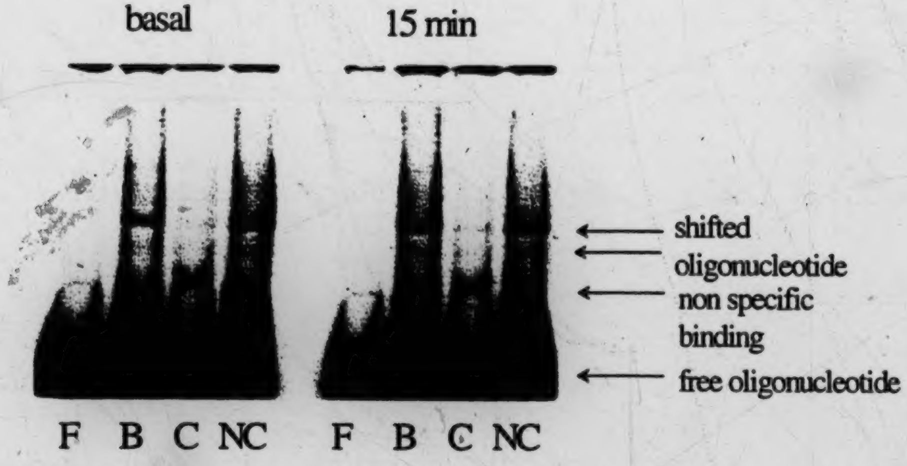

basal
15 min
shifted
oligonucleotide
non specific
binding
free oligonucleotide
F B C NC
F B C NC

**Figure 5.4 Nuclear protein binding to malic enzyme-insulin responsive sequence at basal and at 15 min following oral glucose gavage.** Mice were fasted for 24 hours and then given an oral glucose gavage of 3 g/kg body weight. Mice were sacrificed at 0, 15 and 180 min following oral glucose administration. Liver nuclei were isolated and trancription factor binding to $^{32}$P-labeled ME-IRE probe assessed by gel shift assay. Lanes are labeled as follows: F=Free probe without nuclear proteins added, B=probe with 5 μg nuclear extract added, C=probe with 5 μg nuclear extract added plus 160x excess unlabeled probe to act as competitor DNA, and NC=probe with 5 μg nuclear extract added plus 30 ng of an unrelated DNA sequence to act as non-competitor DNA.

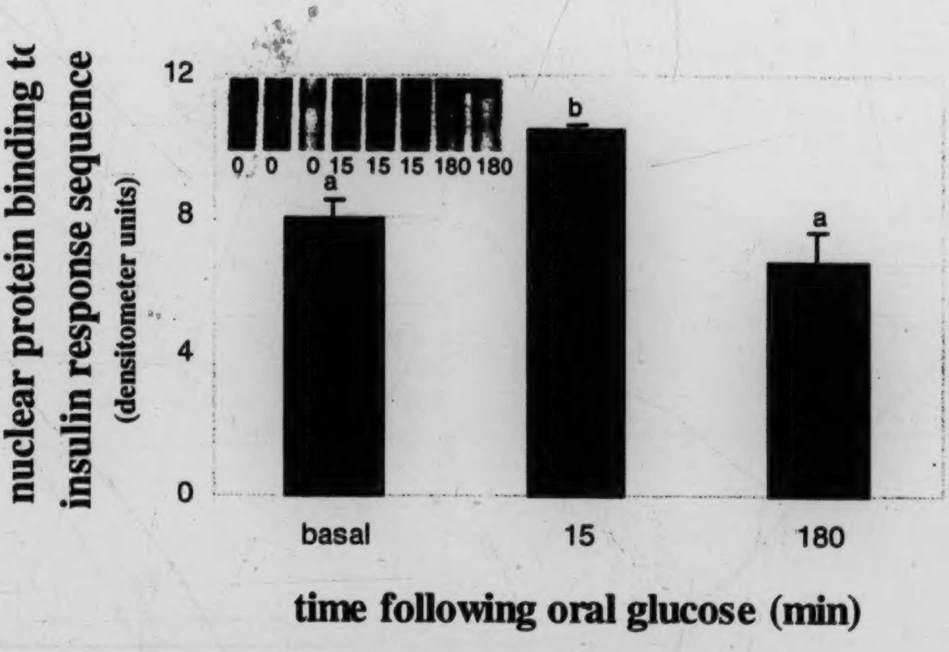


**Figure 5.5 Nuclear protein binding to malic enzyme-insulin responsive sequence at basal and at 15 min following oral glucose gavage**
Mice were fasted for 24 hours and then given an oral glucose gavage of 3 g/kg body weight. Mice were sacrificed at 0, 15 and 180 min following oral glucose administration. Liver nuclei were isolated and trancription factor binding to $^{32}$P-labeled ME-IRE probe assessed by gel shift assay. Bands were quantitated by densitometry and values are expressed in densitometer units. The effect of time following oral glucose treatment on ME-IRE binding was assessed using ANOVA. Values labeled with different letters are significantly different ($p<0.05$). (N= 6 for 0, 15 timepoints, n= 2 for 180 min timepoint)

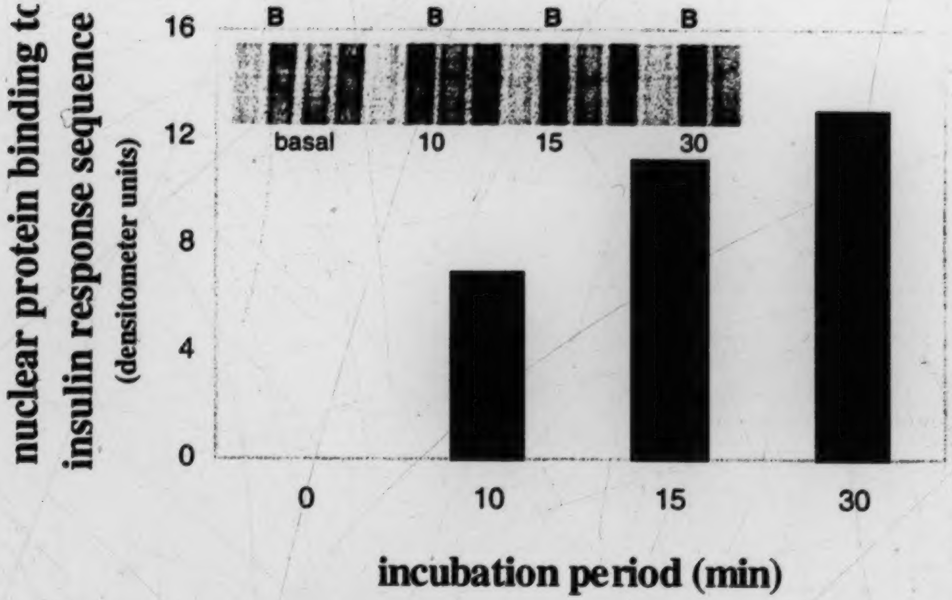


**Figure 5.6 Nuclear protein binding to malic enzyme-insulin responsive sequence at basal and following incubation for different lengths of time with insulin receptor.**

Nuclei were isolated from hepatocytes taken from mice were fasted for 24 h. Insulin receptor were incubated for 10, 15, and 30 min with nuclei extracts prior to adding $^{32}$P-labeled ME-IRE probe. Transcription factor binding was assessed by gel shift assay. Bands in lane labeled (B) represented binding between probe and nuclear extracts with no competitor DNA added. Bands were quantitated by densitometry and values are expressed as a change from basal in densitometer units (0 min = 17.3) This experiment had n = 1.

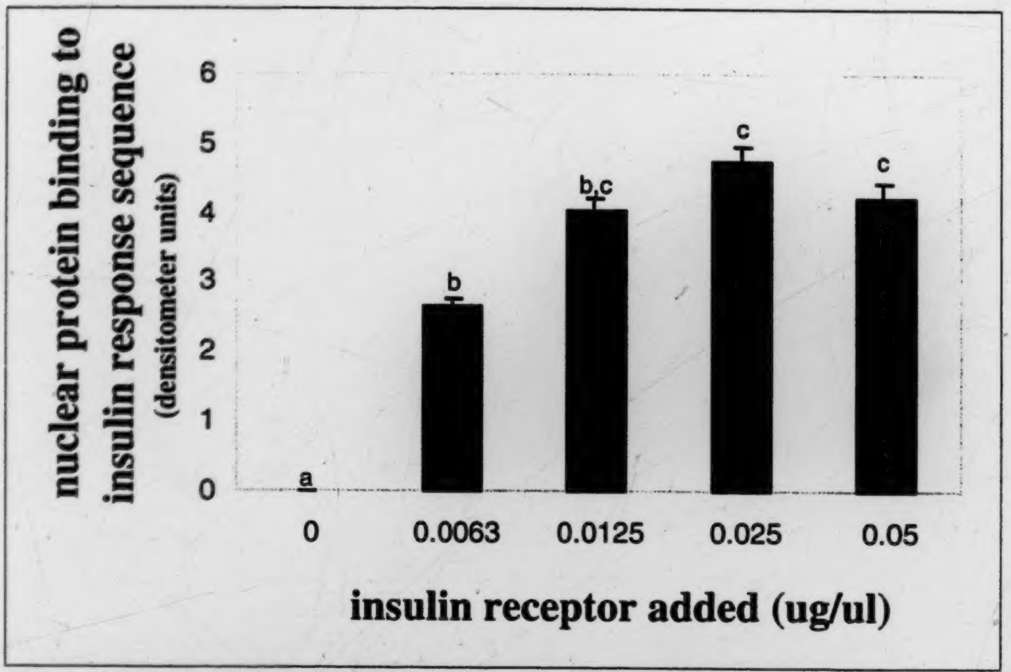


**Figure 5.7 Dose response effect of insulin receptor incubation on nuclear protein binding to malic enzyme-insulin responsive sequence.** Nuclei were isolated from hepatocytes taken from mice were fasted for 24 h. Insulin receptor at varying concentrations, 0.0063, 0.013, 0.025, and 0.05 μg/μl, was incubated for 15 min with nuclei extracts prior to adding $^{32}$P-labeled ME-IRE probe. Transcription factor binding was assessed by gel shift assay. Bands in lane labeled (B) represented binding between probe and nuclear extracts with no competitor DNA added. Bands were quantitated by densitometry and values are expressed as a change from basal in densitometer units (0 = 4.50 ±0.13). The effect of dose of insulin receptor on ME-IRE binding was assessed using ANOVA. Values labeled with different letter are significantly different ($p<0.05$). (N = 5 for each dose of insulin receptor used).

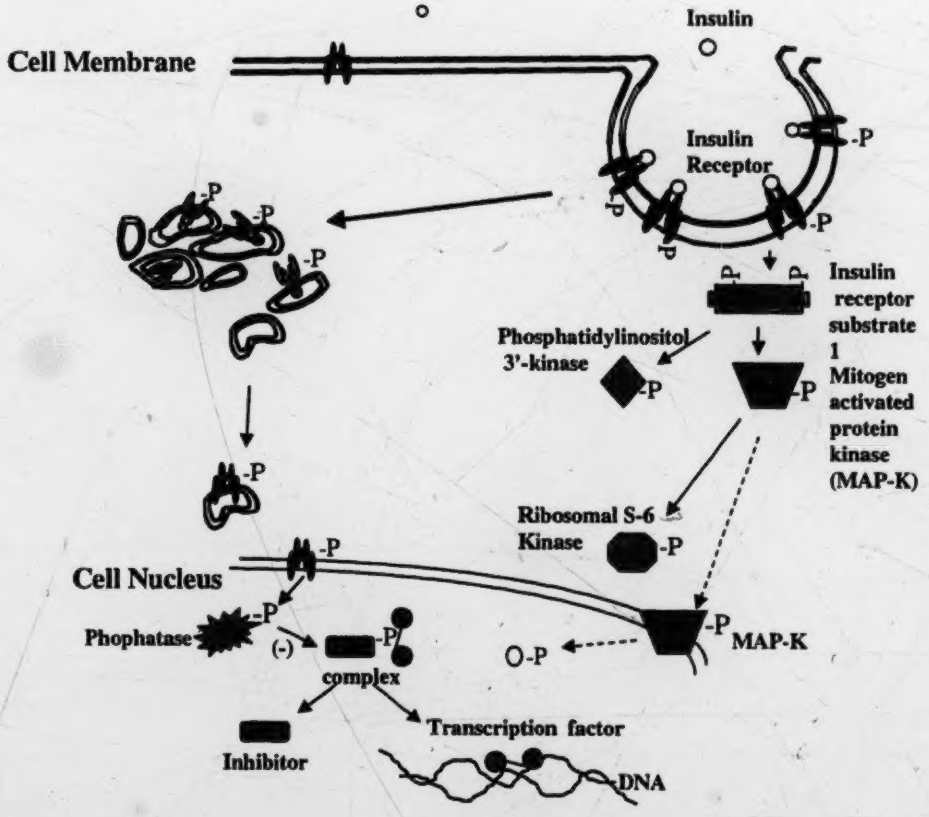

Insulin
Cell Membrane
Insulin Receptor
-P
-P
-P
-P
-P
-P
-P
P
P
Insulin receptor substrate 1
Phosphatidylinositol 3'-kinase
-P
-P
Mitogen activated protein kinase (MAP-K)
-P
Ribosomal S-6 Kinase
-P
-P
Cell Nucleus
-P
Phophatase
(-)
-P
complex
O-P
-P
MAP-K
Inhibitor
Transcription factor
DNA

**Figure 5.8 Proposed model of insulin receptor signaling in the nucleus.**

Insulin binding to the receptor causes β-subunit autophosphorylation and internalization of the receptor tyrosine kinase. Insulin receptor is translocated to the cell nucleus. The insulin receptor phosphorylates a nuclear phosphatase thereby activating its phosphatase activity. The phosphatase removes the phosphate group on an inhibitor protein. Removal of the phosphate group allows the inhibitor protein and a transcription factor to dissociate. The transcription factor is able to bind to the insulin responsive element. Alternatively the phosphatase may remove the phosphate group on a transcription factor which allows it to interact with the DNA. Insulin signal transduction to the nucleus via IRS-1 is outlined.

# VI SUMMARY AND DISCUSSION

The hypotheses tested in this thesis have been verified as follows:

> *Hypothesis 1:* Insulin receptor levels in isolated cell nuclei of hepatocytes taken from fasted animals will increase following an oral glucose meal and an increase in serum insulin concentration.

An oral glucose meal caused an expected rise in serum glucose and insulin concentrations in mice. Insulin receptor concentration increased in the nucleus following an oral glucose meal assessed by insulin binding assay **(Chapter II and Chapter III)** and by immunoassay using anti-insulin receptor antibody **(Chapter IV)**. Peak levels of insulin receptor binding were observed within 30 min of an oral glucose meal. This peak of nuclear insulin binding was coincident with the peak of insulin concentration in serum achieved by oral glucose gavage. Immunoassay of insulin receptor levels in the nuclei revealed that insulin receptor levels in the nucleus were increased at 15 min following oral glucose gavage, and were further increased by 180 min following oral glucose gavage. Differences in the time course of insulin-induced translocation of the insulin receptor may be a reflection of the different methodologies used to measure insulin receptor. Although insulin binding assays are frequently used to measure insulin receptor concentration, this method more likely assesses the ability of the receptor to bind insulin. This property of the insulin receptor is largely dependent on the structure of insulin receptor within its membrane environment. Immunoassay of insulin receptor using anti-insulin receptor involved using polyclonal antibodies prepared against purified insulin receptor. Although the exact epitopes recognized by the antibody preparation were not determined, it is feasible that numerous antibodies bound to the insulin receptor. It is also feasible that antibody binding was less dependent on the ability of the insulin receptor to attain its native structure. Therefore the measurements of the insulin receptor determined by immunoassay would be more likely to approximate receptor content compared to measurements determined by insulin binding assay.

> ***Hypothesis 2:*** Insulin receptor levels in isolated cell nuclei of hepatocytes taken from fasted mice are decreased in obese mice compared to lean animals following an oral glucose meal.

Obese mice had increased levels of insulin receptor in isolated hepatocyte nuclei compared to lean mice following an oral glucose meal. Increased receptor translocation observed in obese mice following insulin stimulation may be due to basal hyperinsulinemia and the dramatic increase in serum insulin induced by an oral glucose meal compared to lean mice. Increased ligand binding due to hyperinsulinemia may increase insulin receptor internalization in obese mice.

Alternatively insulin receptor recycling back to the plasma membrane may be impaired in obesity. No differences were observed in insulin binding ability of nuclear receptors of lean and obese animals. The increased amount of insulin receptor detected in nuclei from obese mice was not measured by insulin binding assay. This finding may suggest the insulin binding function of the nuclear receptor within the hepatocyte nuclear membrane is functionally impaired. It was hypothesized that insulin receptor translocation to the nucleus of obese mice would be impaired. Evidence suggests that obesity is associated with reduced insulin binding to plasma membrane receptors resulting from receptor downregulation. Altered membrane composition has been suggested to decrease nuclear insulin binding in obese mice compared to lean mice fed high fat diets. Further studies are required to determine whether despite increased translocation, insulin receptor function in the nucleus is impaired.

> ***Hypothesis 3:*** Insulin receptor translocation to the nucleus following an oral glucose meal is reflected in an increase in nuclear insulin receptor β-subunit autophosphorylation.

The appearance of tyrosine-phosphorylated insulin receptor β-subunit was increased in the hepatocyte nucleus within 15 min of an oral glucose meal. Although basal levels of insulin receptor were detected in the nucleus, no tyrosine phosphorylation was detected in mice in the basal state. This finding suggests that insulin receptor in the nucleus is not activated in the basal state and that activity of the insulin receptor tyrosine kinase in the nucleus is dependent upon ligand-induced internalization and nuclear translocation of the cell surface insulin receptor. Insulin receptor detected in the nucleus in the basal state may reflect newly synthesized insulin receptor en route to the plasma membrane.

> ***Hypothesis 4:*** Insulin receptor translocation to the nucleus following an an oral glucose meal is coincident with increases in tyrosine phosphorylation of nuclear proteins.

An oral glucose meal decreased the tyrosine phosphorylation of a 30 KDa nuclear protein within the time course of the appearance of insulin receptor tyrosine kinase in the hepatocyte nucleus. As the insulin receptor is a tyrosine kinase, increases in tyrosine phosphorylation of nuclear proteins were expected upon translocation of the insulin receptor to the nucleus. The 30 KDa protein does not appear to be a direct substrate of the nuclear insulin receptor tyrosine kinase. Its dephosphorylation may be catalyzed by a nuclear phosphatase whose action is regulated by the nuclear insulin receptor tyrosine kinase. Insulin stimulation of intact cells has been demonstrated to induce the dephosphorylation of nuclear proteins.

> ***Hypothesis 5:*** Insulin receptor translocation to the nucleus following an

> oral glucose meal is coincident with increases in expression of insulin-responsive genes including malic enzyme, fatty acid synthase and glyceraldehyde 3-phosphate dehydrogenase.

An oral glucose meal induced a transient increase in insulin-responsive genes, malic enzyme and glyceraldehyde 3-phoshate dehydrogenase mRNA levels. Peak increases in mRNA levels occurred within 15 min following insulin stimulation and the appearance of insulin tyrosine kinase in the nucleus. The time required for observing insulin-induced gene expression upon insulin stimulation *in vivo* was more rapid than has been previously reported. Increases in leptin and pyruvate kinase gene expression were observed only after 2 h following insulin stimulation in the intact animal. Stimulation of cells in culture with insulin produced increases in expression of c-fos and PEPCK genes within 5 and 30 min respectively. In this study, changes in FAS gene expression were not observed within 180 min of an oral glucose meal. The differences in time required for the insulin regulation of gene expression may reflect different mechanisms involved in insulin signaling to the nucleus. For some genes the insulin response may require *de novo* protein synthesis or a combination of nutrients and growth factors.

> ***Hypothesis 6:*** Insulin receptor translocation to the nucleus following an oral glucose meal is coincident with increases in transcription factor binding to an insulin responsive sequence.

Transcription factors present in isolated hepatocyte nuclei exhibited increased binding to the malic enzyme insulin response sequence following an oral glucose meal. Increased binding affinity of IRE binding proteins following insulin stimulation *in vivo* occurred in parallel to increases observed in mRNA levels. This finding confirms that insulin regulation of malic enzyme gene expression involves modulation of transcription factor association with the malic enzyme IRE. De novo protein synthesis is unlikely to occur within 15 min of insulin stimulation via an oral glucose meal. IRE binding proteins were detected in the basal state in the hepatocyte nucleus. Increases in protein-IRE complex formation following an oral glucose meal were probably caused to post-translational modification of the constitutive IRE-binding proteins.

> ***Hypothesis 7:*** Direct incubation of insulin receptor with cell nuclei isolated from hepatocytes taken from fasted animals will increase phosphorylation of DNA-binding nuclear proteins.

Changes observed in the hepatocyte nucleus upon insulin stimulation *in vivo* were coincident with the appearance of activated insulin receptor in the nucleus. These changes included a decrease in tyrosine phosphorylation of a 30 KDa nuclear protein, an increase in transription factor binding to the malic enzyme IRE and an increase in malic enzyme gene expression. To determine if newly translocated insulin receptor in the hepatocyte nucleus could be involved in directing insulin nuclear signaling, an experiment was designed to examine insulin receptor signaling in the hepatocyte nucleus in the absence of insulin receptor substrate-1-mediated intracellular signaling. Insulin receptor incubation with DNA-binding protein attenuated the incorporation of [$^{32}$-P]-phosphate into a 30 KDa and a 37 KDa protein. Insulin receptor-induced inhibition of a nuclear kinase or activation of a nuclear phosphatase may be responsible for preventing the labeling of the 30KDa and the 37 KDa proteins. A similar insulin-induced decrease in the phosphorylation state of a 30 KDa DNA-binding protein following *in vivo* insulin stimulation was observed as was seen following direct incubation of nuclear protein with insulin receptor. These observations suggest that the 30 KDa DNA binding protein may lie downstream of the nuclear insulin receptor signal. Future studies should attempt to identify this protein as well as putative endogenous substrates of the nuclear insulin receptor.

***Hypothesis 8*:** Direct incubation of insulin receptor with cell nuclei isolated from hepatocytes taken from fasted animals will increase the binding of transcription factors to an insulin responsive sequence.

Regulation of transcription factor function can be mediated via changes in phosphorylation state. Incubation of isolated nuclear proteins taken from the hepatocytes of fasted mice with activated insulin receptor increased transcription factor binding to malic enzyme IRE. It was established that *in vivo* stimulation with insulin resulted in increased transcription factor-IRE association. This finding suggests that increased binding of IRE to specific nuclear proteins can be induced *in vitro* by direct incubation of nuclear extract with insulin receptor. Within this closed system there is an absence of indirect cytoplasmic-to-nucleus signaling via the insulin receptor substrate-1. Based upon our findings that insulin induced a decrease in phosphorylation of a nuclear protein it may be suggested that the association of IRE to its transcription factor is increased upon dephosphorylation of the latter. Future studies should attempt to determine the mechanism of insulin receptor regulation of transcription factor binding to the malic enzyme IRE.

Insulin has long known to regulate the expression rate of genes, however the mechanim used in insulin signaling to the nucleus remain to be elucidated. The role of insulin receptor is to deliver the insulin signal from the cell surface to various mediator proteins and then effector proteins within the cytoplasm and nucleus. The insulin receptor substrate family of proteins becomes rapidly phosphorylated by insulin receptor and then stimulates activation of other proteins (1). Few other cytoplasmic proteins have been characterized whose phosphorylation is catalyzed directly by the insulin receptor tyrosine kinase (2). This evidence suggests that insulin signaling to the nucleus be directed through insulin receptor substrate proteins. This thesis proposes an alternate mechanism of insulin signaling to the cell nucleus, via direct interaction with and activation of proteins within the nucleus by the insulin receptor.

Insulin receptor on the cell surface internalizes immediately following binding to insulin. It was hypothesized that internalization and translocation of the insulin receptor may bring the insulin receptor tyrosine kinase within close proximity of nuclear proteins. This research provides evidence that insulin receptor is translocated to the nucleus within 15 min of a glucose meal. The rapid time course at which insulin receptor translocation is observed *in vivo* suggests that direct signaling to the nucleus is a rapid pathway by which insulin is able to regulate nuclear proteins. The stimulation of insulin receptor substrate-1 activation by insulin *in vivo* is reported to be maximal within 30 sec (1). Time courses for activation of other intermediates in the insulin signal transduction pathway is slower. In cultured cells maximum stimulation of MAP-K and Shc occurs at 5 min (3,4) and maximum stimulation of P-I-3'K occurs at 3 min (5). Thus for some nuclear process direct signaling via nuclear insulin receptor may be preferred to signaling mediated by cytoplasmic proteins activated by insulin receptor on the cell surface.

Although a putative substrate of the insulin receptor tyrosine kinase in the nucleus was not identified, increased nuclear phosphatase activity following insulin stimulation and incubation of nuclear proteins with insulin receptor was evident. Direct involvement of the insulin receptor in mediating transcription factor binding to cis-acting elements was observed. These findings are novel in this area of research. These observations present strong evidence that the insulin receptor is directly responsible for regulating insulin responses in the nucleus as they occurred in the absence of insulin mediated cytoplasmic signaling. Future studies should identify which nuclear proteins are substrates of the nuclear insulin receptor tyrosine kinase, whether these proteins have tyrosine phosphatase activity. It will also be important to identify transcription factors downstream of the signal transmitted by the insulin receptor in the nucleus.

Fasting and refeeding induced insulin receptor translocation and insulin-responsive responses in the cell nucleus. This finding is significant as it suggests that this novel pathway, by which insulin regulates nuclear processes, functions under physiological conditions such as following a high carbohydrate meal. In the present study, increased insulin receptor translocation to the nucleus was observed in the obese animal. One aspect of the pathology of obesity includes increased expression of lipogenic genes (6). Future studies should determine if faulty regulation of insulin signal transduction to the nucleus via insulin receptor translocation may explain some aberrations that occur in obesity.

## *REFERENCES*

1. Kahn, C.R., White, M.F., Shoelson, S.E., Backer, J.M., Araki, E., Cheatham, B., Csermely, P., Folli, F., Goldstein, B.J., Huertas, P., Rothenberg, P.L., Saad, M.J.A., Siddle, K., Sun, X-J., Wilden, P.A., Yamada, K., and Kahn, S.A. The insulin receptor and its substrate: molecular determinants of early events in insulin action. (1993) Rec. Prog. Horm. Res.,48:291-229.

2. Myers, M.G., and White, M.F. Insulin signal transduction and the IRE proteins. (1996) Ann. Rev. Pharmacol. Toxicol.,36:615-658.

3. Sasaoka, T., Ishiki, M., Sawa, T., Ishihara, H., Takata, Y., Imamura, T., Usui, I., Olefsky, J.M., and Kobayashi, M. Comparison of the insulin and insulin-like growth factor 1 mitogenic intracellular signaling pathways. (1996) Endocrinol.,137:4427-4434.

4. Begum, N., Ragolia, L., and Srinivasan, M. Effect of tumor necrosis factor-alpha on insulin-stimulated mitogen-activated protein kinase cascade in cultured rat skeletal muscle cells. (1996) Eur. J. Biochem.,238:214-220.

5. Levy-Toledano, R., Taouis, M., Blaettler, D.H., Gorden, P., and Taylor, S.I. Insulin-induced activation of phosphatidylinositol 3-kinase. Demonstration that the p85 subunit binds directly to the COOH terminus of the insulin receptor in intact cells. (1994) J. Biol. Chem.,269(49):31178-31182.

6. Dugail, I. Quignard-Boulange, A., Le Liepvre, X., and Ardouin, B. Gene expression of lipid storage-related enzymes in adipose tissue of the genetically obese Zucker rat: coordinate increase in transcriptional activity and potentiation by hyperinsulinemia. (1992) Biochem. J.,282(3):607-611.

# APPENDIX

**Appendix I Insulin receptor purification from rat liver plasma membrane.**

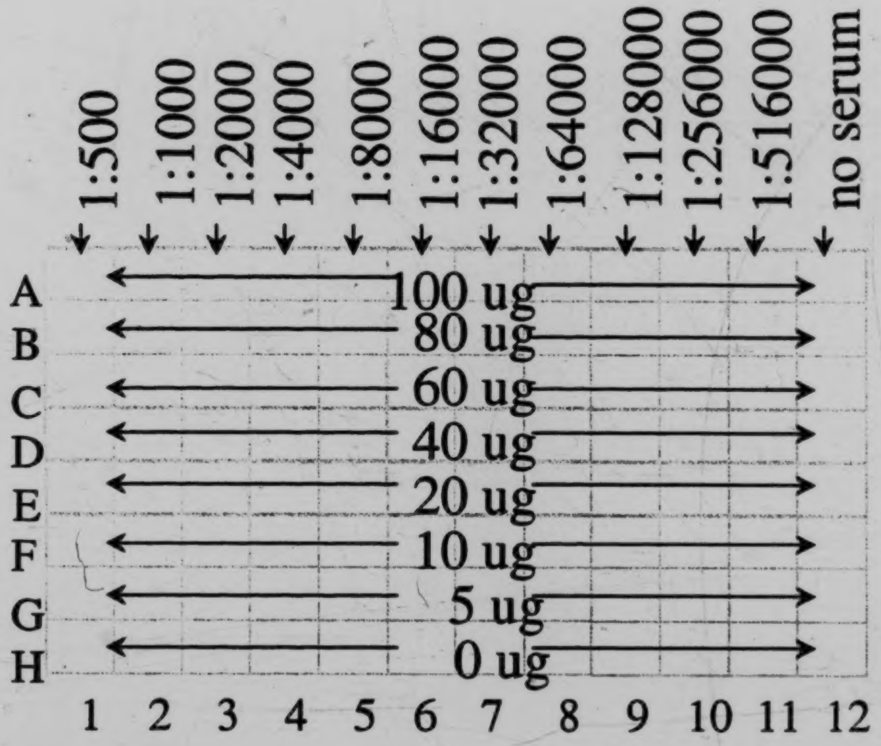
| | Total Protein (mg) | Insulin Binding (fmol/mg protein) | Total Insulin Binding (fmol) | Yield (%) | Purification Factor |
|---|---|---|---|---|---|
| Plasma Membrane | 11.75 ± 1.82 | 7.43 ± 1.00 | 82.16 ± 6.82 | 100 | |
| Solubilized Plasma Membrane | 7.22 ± 1.38 | 6.96 ± 0.73 | 51.39 ± 13.75 | 60 ± 11 | 0.98 ± 0.13 |
| Wheat Germ Agarose Eluent | 1.40 ± 0.34 | 23.1 ± 5.40 | 36.90 ± 16.58 | 41 ± 15 | 3.11 ± 0.58 |
| Insulin Agarose Eluent | 0.03 ± 0.01 | N.D. | N.D. | N.D. | N.D. |

200 KDa
97 KDa
66 KDa
45 KDa
30 KDa
Alpha subunit
Beta subunit
1
2
3

**Appendix II Iodination of insulin-agarose eluent.**
10 µg of insulin-agarose eluent was used for iodination assay. Protein was incubated in a total of 50 µl phosphate buffered saline pH 7.4 containing 0.1 % (v/v) Nonidet P-40 (Sigma, St. Louis, MO, USA) (PBS-NP40) and 3 Iodo-Beads (Pierce, Rockford, IL, USA) for 5 min at room temperature. 500 µCi of $^{125}$-I was added to mixture and allowed to incubate for 10 min at room temperature. Mixture was then added to a PD-10 column (Pharmacia, Biotech. Inc., Baie d'Urfe, Quebec, Canada) pre-equilibrated in PBS-NP40. 1 ml fractions were collected and counted in a Gamma 8000 counter (Beckman Instruments, Palo Alto, CA, USA). Fractions 3 to 5, which was the first radioactivity containing peak, was pooled. A 15 µl sample was boiled in Lamelli sample buffer, boiled for 5 min and then electrophoresed using SDS-PAGE (7.5 % (w/v) acrylamide) at 25 mA. Bands were visualized by autoradiography.

Lanes are labeled as follows: MW= molecular weight standards, 1, 2 and 3 = 4 µl, 8 µl, and 12 µl sample loaded onto gel. (Some of sample from lane 1 spilled into next lane).

The visible band between 66 and 45 KDa could not be identified as an insulin receptor subunit and may either be a breakdown product or contamination.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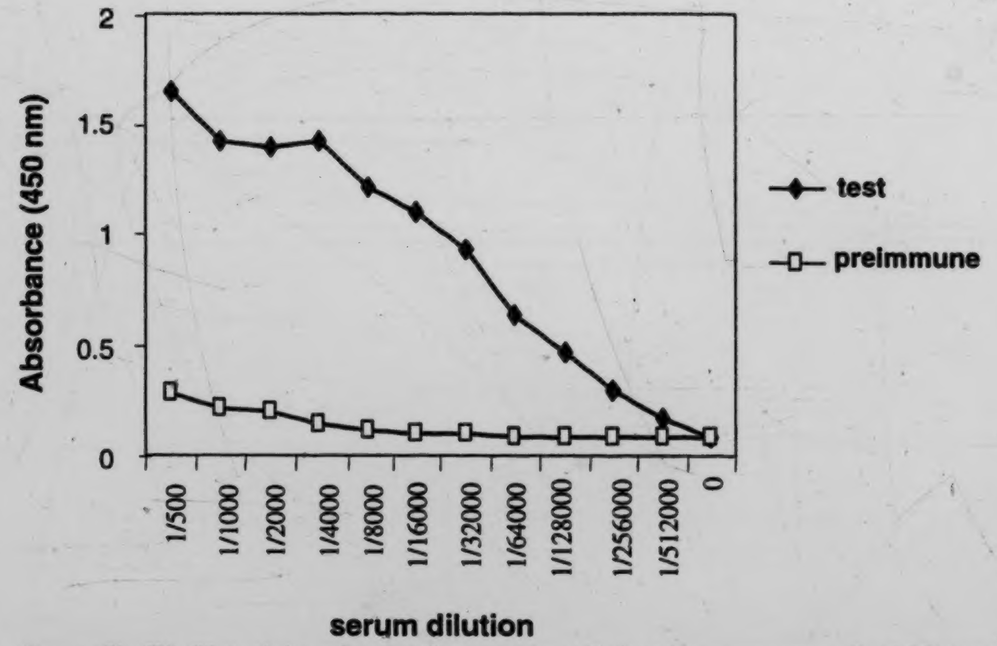
1:4000
1:8000
1:16000
1:32000
1:64000
1:128000
1:256000
1:516000
no serum
A
B
C
D
E
F
G
H
100 ug
80 ug
60 ug
40 ug
20 ug
10 ug
5 ug
0 ug
1
2
3
4
5
6
7
8
9
10
11
12

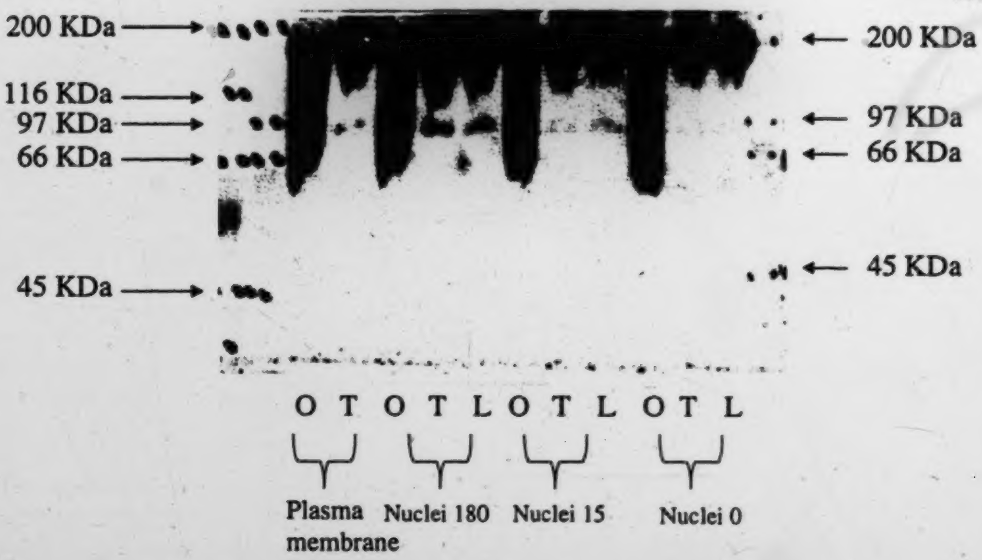
**Appendix III Enzyme linked immunoassay of rabbit test serum and rabbit background serum.**

The 96-well microtitre plates (Dynatech Immulon 2) were coated with 200 µl per well of phosphate buffered saline pH 7.3 containing 100, 80, 60, 40, 20, 10, 5 and 0 µg of plasma membrane isolated from rat liver membrane as shown in the table above. Plates were stored at $4^0$C overnight in a plastic bag. The next day the solution was shaken from the plate and each well coated with 200 µl of 1% (v/v) bovine serum albumin (Sigma, St. Louis, MO, USA), for 1 h at room temperature. Wells were washed with PBS containing 1 % (v/v) Tween 20 (Sigma), (PBS-T). Rabbit serum was diluted 1:500 with PBS-T added to the first of the 12 lanes of the microtitre plate (200 µl well). Successive 1/2 dilutions were made in each of the following 10 lanes as shown in the table below. Plates were incubated with antibody for 2 h at room temperature. The plates were washed with PBS-T. Goat anti-rabbit diluted 1:3000 in PBS-T was added and incubated for 2 h at room temperature. Plates were washed with PBS-T. Color was developed with *o*-phenylenediamine (0.4 mg/ml), and urea peroxidase (1 mg/ml) in 0.1 M citrate buffer (pH 4.75) added to each well (200 µl/well). Plates were read using an ELISA reader at $A_{450}$. Serum dilution vs absorbance is plotted in **Appendix IV**



**Appendix IV Plot of absorbances from enzyme linked immunoassay of rabbit test serum.**

Absorbance readings (450 nm) obtained from rat plasma membrane tested with serial dilutions of rabbit preimmune and test serum as outlined in **Appendix III**. Only results obtained from 5 µg rat plasma membrane are graphed, n = 1.



**Appendix V Immunoprecipitation of insulin receptor in plasma membrane and nuclei using antiserum prepared in laboratory and commercially available antibodies.**

Plasma membrane (100 μg protein) and nuclei extract (300 μg protein) was precleared three times each with rabbit serum agarose (Sigma) and then with recombinant Protein A -agarose (Upstate Biotechnology Inc.) and then incubated with 5 μg anti-insulin receptor overnight at 4$^{0}$C. Recombinant Protein A-agarose (100 μl of a 50% (v/v) suspension) was added to the mixture and incubated for 2 h at 4$^{0}$C with rotation. Samples were then centrifuged for 1 min at 14 000 x g and the pellet containing anti-insulin receptor-antigen-agarose conjugate was washed three times in a PBS pH 7.3 buffer supplemented with 1% (v/v) Triton X-100, 0.5% (w/v) soduim deoxycholate, 0.1% (w/v) SDS, 0.04 μg/ml NaF, and then twice with PBS, 0.04 ug/ml NaF. The washed beads were resuspended in 50 μl of Laemmeli sample buffer (34) with 5% (v/v) 2-mercaptoethanol, boiled for 5 minutes, and centrifuged for 5 minutes at 14 000 x g; the supernatent was then analyzed on 7.5% (w/v) SDS-PAGE followed by western blotting with anti-PY. Lanes are labeled as follows: O=Oncogene GRO7 anti-insulin receptor antibody, T=Transduction Laboratories anti-insulin receptor antibody, L=laboratory produced anti-insulin receptor antibody. Nuclei 180=nuclei 180 min, Nuclei 15= nuclei 15 min, and Nuclei 0= nuclei 0 min following oral glucose gavage.

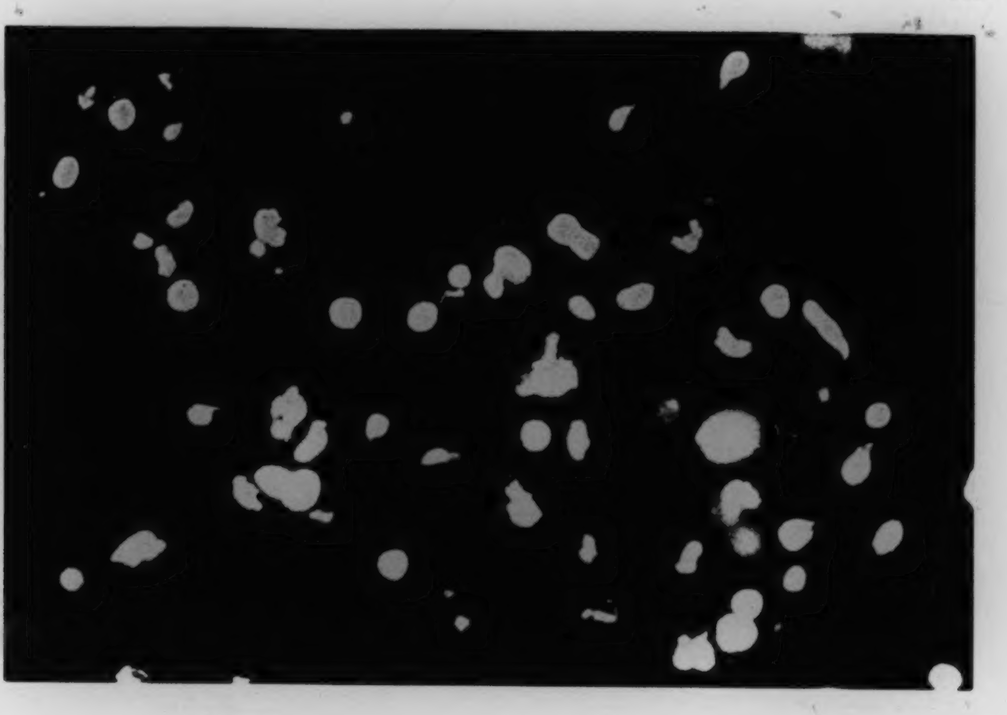
## Appendix VI Nuclei isolated from hepatocytes in buffers containing low and high phosphatase inhibitors.

Nuclei suspensions were inspected visually under the light microscope after staining with methylene blue (0.2% v/v) and ethidium bromide (100 μg/ml) at 400 x magnification using a Dialux 20 EB microscope, Leitz, Berlin, Germany.

Key:

1: Nuclei isolated in buffers not containing phosphatase inhibitors stained with ethidium bromide.
2: Nuclei isolated in buffers not containing phosphatase inhibitors stained with methylene blue.
3: Nuclei isolated in buffer containing low phosphatase inhibitors stained with ethidium bromide.
4: Nuclei isolated in buffer containing low phosphatase inhibitors stained with methylene blue.
5: Nuclei isolated in buffer containing high phosphatase inhibitors stained with ethidium bromide.
6: Nuclei isolated in buffer containing high phosphatase inhibitors stained with methylene blue.

1



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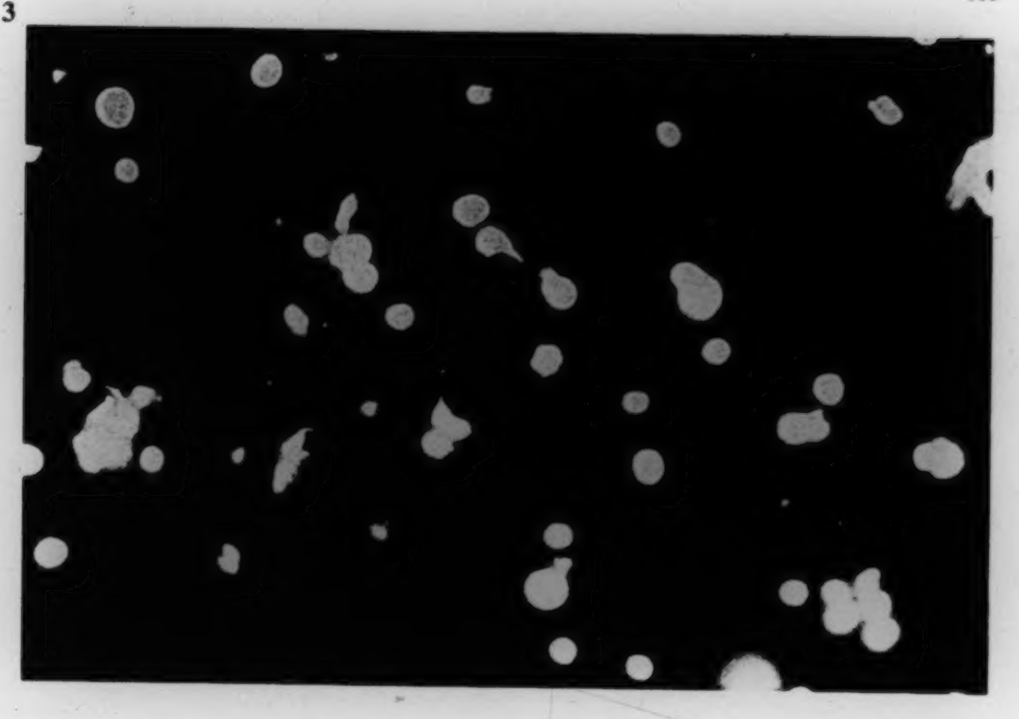
3



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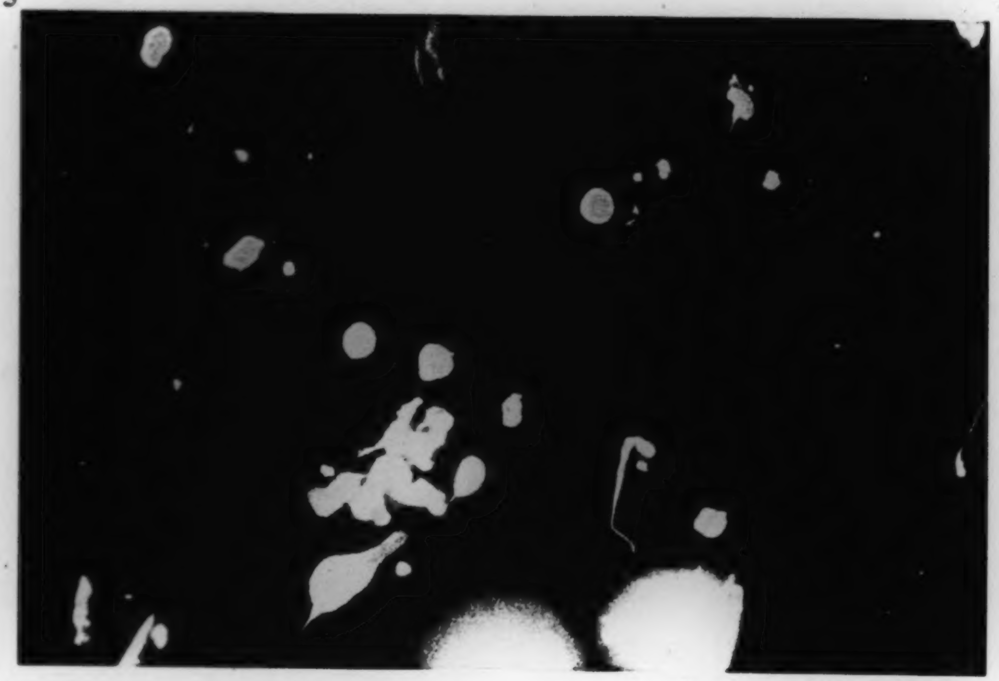

6